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Part II

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RAPID BIOCHEMICAL TECHNIQUE FOR PHYTOTOXICITY MODES-OF-ACTION OF HERBICIDES

Part II

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CHAPTER I

GENERAL INTRODUCTION

By Fumihiko Hayashi and Hiroshi Ikuma

A. OVERALL CONSIDERATIONS

The research project reported herein is the second of the two year project, sponsored by the U. S. Environmental Protection Agency under the Substitute Chemicals Program, and is ultimately aimed at developing a rapid screening technique to determine the modes-of-action of herbicides.

The first year project emphasized the analyses of biochemical action of herbicidal phytotoxicity on plant cellular processes and those of herbicidal effect on ultrastructural alterations. These investigations were carried out with isolated organelles (mitochondria and chloroplasts) and isolated tissues (root tips and leaf disks). These studies gave rise to the following observations: (a) a herbicide may act on more than one metabolic processes; (b) the conclusions derived from the work with isolated organelles do not necessarily predict the herbicidal effects on the activities of isolated tissues; (c) herbicides tend to inhibit protein and nucleic acid syntheses indirectly; and (d) many herbicides act to alter cellular ultrastructures particularly of root tips. Thus, in search for primary modes of action of herbicides, the use of isolated organelles and isolated tissues may not necessarily be ideal, while ultrastructural analyses, though not sufficient by themselves, would shed important light on herbicidal action.

Clearly, conclusions obtained from the studies with isolated organelles lose their significance, unless they are confirmed by studies with intact plants, or vice versa. Based on these considerations of the first year, the second year projects were formulated into the following three tasks:

Task 1: Development of Simultaneous Measurements of Plant Processes

Task 2: Analysis of Ultrastructural Changes

Task 3: Examination of Organelles in Treated Tissues

These investigations were carried out using the same plant, i.e., young growing soybean plant. The major emphasis was placed on the development of simultaneous measurements of important plant processes (Task 1), while Tasks 2 and 3 were performed to aid further analyses of biochemical modes of action of herbicides.

B. GENERAL PLAN OF INVESTIGATION

The overall plant processes can be conveniently considered to consist of the activities primarily associated with the shoot and those primarily associated with the root (Fig. I-1). The major process in the shoot can be ascribed to photosynthesis in the leaves and that in the root, respiration. Photosynthesis in the leaves provides the necessary substrates not only for the growth and maintenance of the shoot tissues, but also for the respiration and growth of the root tissues. The main function of respiration is to provide cellular energy for energy-requiring processes. The root respiration not only supports the processes associated with the growth of the root tissues, but also provides cellular energy needed for active transport of mineral ions from the soil. The mineral ions taken up by the root in turn facilitates osmotic uptake of water by the root from the soil water. Water and essential minerals taken up by the root are distributed throughout the plant with the help of the transpirational pull by the leaves. Transpiration, or the evaporation of water through tiny pores (called stomata) on the leaf surface, provides not only the means for transport of water and minerals from the root to the shoot, but also the means for exchange of

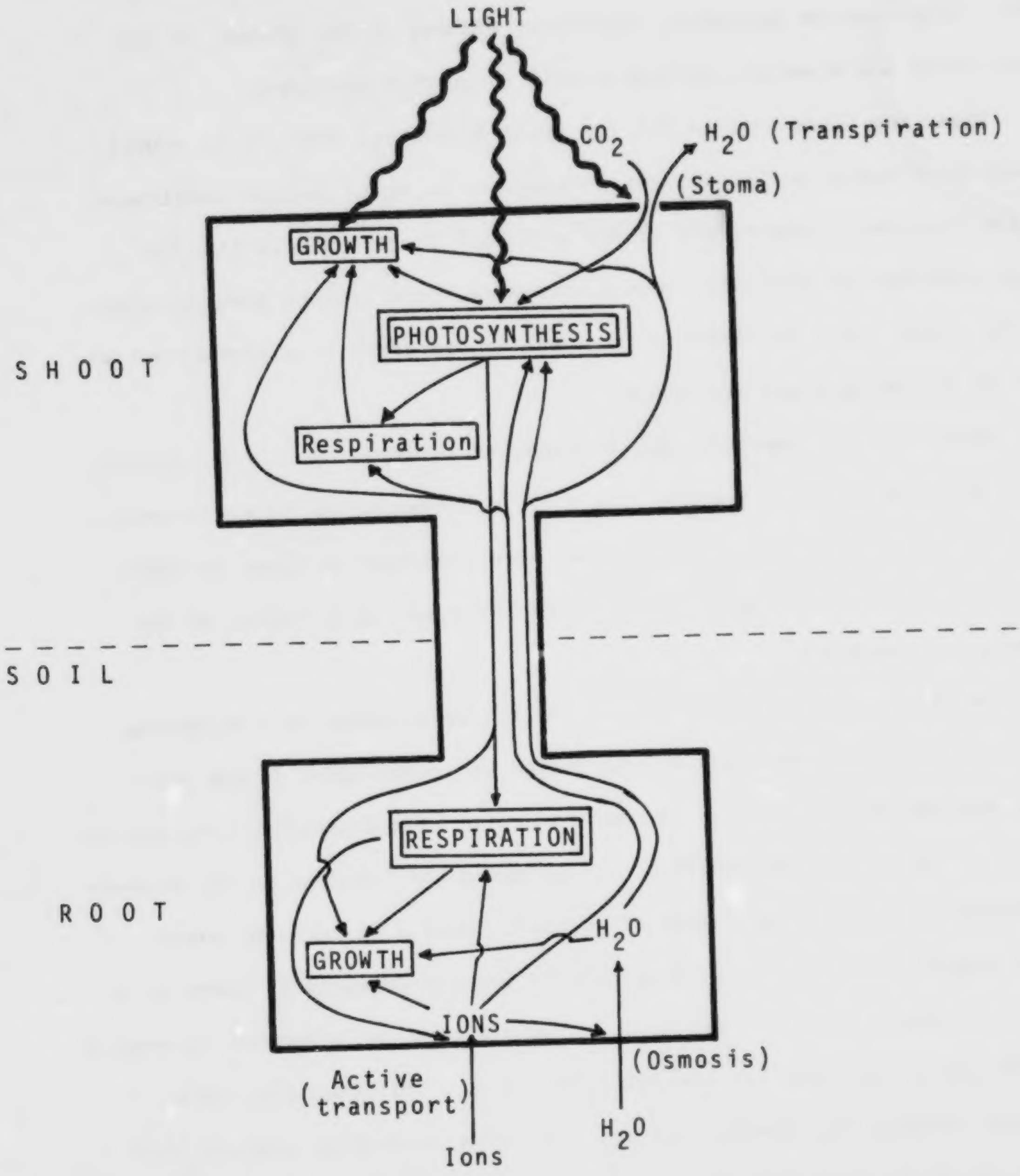

LIGHT
CO_2
H_2O (Transpiration)
(Stoma)
GROWTH
PHOTOSYNTHESIS
SHOOT
Respiration
SOIL
RESPIRATION
ROOT
H_2O
GROWTH
IONS
(Osmosis)
(Active transport)
H_2O
Ions

Fig. I-1. Diagramatic presentation of major processes in plants.

gases (CO_2 and O_2) through the stomata between the plant and the environment. Light exerts important, regulatory effects on the growth of the plant shoot and stomatal opening as well as on photosynthesis.

Thus, the plant carries out its vital activities not only in a well coordinated manner within it, but in response to environmental conditions. If the coordinated activities of the plant are disturbed more than its vital capacity can tolerate, such disturbance would lead to eventual death of the plant. Such disturbances are usually expressed in disturbed activities of the major plant processes.

Herbicides are chemical agents which, if absorbed and not detoxified, would kill the plant. In search for a rapid screening technique to determine the modes-of-action of herbicides, attention must be given to rapid detection of the disturbance, incurred by herbicide application, of the major plant processes.

The major plant processes chosen for the development of a screening technique were photosynthesis in the leaves and respiration in the root. Since photosynthetic activity of the leaf cells is influenced by the availability of CO_2 to the chloroplasts, a hygrometer was inserted in our measuring system to measure the degree of stomatal opening in terms of water vapor release from the leaf. Root respiration was measured by means of an oxygen electrode and a CO_2 electrode. In addition, we attempted to measure mineral ion uptake with ion specific electrodes. Unfortunately, such attempts resulted in repeated failure. In order to examine possible problems associated with instrumentation, leaf photosynthesis and root respiration were separately analyzed (CHAPTERS II and III). After the instrumentation was established, both techniques were combined to make simultaneous measurements of both shoot and root processes (CHAPTER IV).

For these studies, young soybean plants initially grown in the soil were transferred to a solution culture condition. This transfer was in part necessitated by the measuring technique of the root processes and in part to make uniform, rapid additions of herbicides. The solution culture condition allowed the plant to be in a well watered condition. Plants conditioned in this manner were used for electron microscopic studies as well.

A possible use of the chlorophyll fluorescence technique was suggested from a preliminary series of work of the first year to analyze phytotoxicity of herbicide on photosynthesis. This work was continued in the second year, in the hope of adding this technique to the combined technique to measure plant processes. The investigations with diallate and trifluralin indicated problems in assessing their phytotoxicity; the idea of adding a fluorescence device to the combined instrumentation was discouraged (CHAPTER V).

During the first year, electron microscopic studies were performed with a conventional, time-consuming procedure of thin section preparation. In order to quicken the preparatory steps, a new procedure of Muller and Jacks was examined and adopted for the work of the current year. In addition, this work was performed with plants to the root of which herbicides were given, rather than treating plant tissues with herbicides (CHAPTER VI).

Analyses of the properties of organelles from herbicide treated tissues were prompted by our work of the first year when electron microscopic studies revealed drastic alterations of organelle ultrastructure by herbicides. This work posed technical problems of proper separation of altered organelles. An extention of this work, however, revealed an interesting phenomenon in that diallate stimulated aggregation of isolated mitochondria (CHAPTER VII).

In this report, Task 1 work is reported in CHAPTERS II-V, Task 2 investigations in CHAPTER VI, and Task 3 studies in CHAPTER VII. Only a limited number of references are referred to in this report, because the majority of information is the outgrowth of the first year's project whose report include a large number of references. The same herbicides as those used in the preceding year were used for the investigations reported herein. In these studies, soybean plants (*Glycine max* cv. Anoka) were almost exclusively used.

C. SUMMARY OF THE RESULTS

Task 1: Development of Simultaneous Measurements of Plant Processes

Using an infrared CO_2 gas analyzer and a hygrometer, an instrument was constructed simultaneously to measure both mesophyll photosynthesis (measurement of CO_2 uptake) and stomatal opening (analysis of water vapor concentration) with a single leaflet of a soybean plant. To obtain an optimal performance, however, plants must to preconditioned in diffuse light overnight. This instrument was semiautomated. From the time course analysis of herbicidal effect, it can readily be deduced whether a given herbicide acts primarily to inhibit mesophyll photosynthesis or the mechanism of stomatal opening. As predicted from the work of the preceding year, most herbicides tested inhibited mesophyll photosynthesis before inducing stomatal closure. It was of interest to observe that amitrole whose inhibitory action was only very weakly detected in the first year's assays induced stomatal closure at about the same time as its inhibition of mesophyll phytosynthesis.

Repeated, unsuccessful attempts were made to simultaneously analyze root respiration (with both an O_2 and CO_2 electrodes) and ion uptake activity (by means of ion specific electrodes). Due to biological and instrumental problems, we had to abandon the use of the ion specific electrodes. The

O_2 and CO_2 electrodes provided expected results in a manner similar to those obtained in the first year with isolated corn root tips.

A successful attempt was made to combine the analyses for both the shoot processes and the root processes. For this purpose, both a gas analyzer and a hygrometer were used for the shoot analysis, but only an O_2 electrode was used for root respiration analysis. A CO_2 electrode was eliminated from the analysis of the root processes, because the results from the CO_2 electrode had been practically identical with those from the O_2 electrode and also the CO_2 electrode exhibited a longer time constant and less stability than the O_2 electrode. The combined instrument was tested with three types of herbicides; a herbicide primarily inhibiting mesophyll photosynthesis, a herbicide primarily inhibiting root respiration, and a herbicide inducing stomatal closure. Time course responses of the plants to these herbicides were exactly what had been predicted. The instrumentation of this type would be successfully used for a routine herbicide screening procedure.

When the same leaf disk fluorometer as the one used in the first year was employed to examine the effects of diallate and trifluralin on chlorophyll fluorescence, anomalous results were obtained; i. e., diallate addition produced much higher fluorescence than diuron control, and trifluralin did not cause any change in fluorescence. The nature of these anomalies was further studied with isolated spinach chloroplasts. Diallate induced fluorescence data do not agree with similar data obtained with the O_2 electrode; diallate appears to cause multiple effects. Trifluralin acts to quench fluorescence, even though it acts to inhibit electron transport between photosystems I and II.

Task 2: Analysis of Ultrastructural Changes

In order to shorten the preparatory period of electron microscopy, we attempted to adopt a rapid method of dehydration with 2,2-dimethoxypropane and of embedding with Spurr's low density epoxy resin, recently published by Muller and Jacks (1975). With this method, the preparatory time was halved and the quality of electron photomicrographs was equal or somewhat better contrasting.

Young soybean plants which had been transferred to a solution culture condition was given a herbicide for 2 hours and tips of secondary roots and leaf pieces were used for the ultrastructural study. Similar to the investigations of the preceding year, root tip cells showed much more pronounced effects of herbicides than leaf cells which showed practically no effect of herbicidal alteration of ultrastructure. Herbicidal alterations of cellular ultrastructures in root tips appeared in organelle morphology, mitotic activity, vacuolar morphology, and cell wall morphology, in a manner similar to the study of the preceding year. Of the 16 herbicides tested, 9 herbicides showed less effects than the preceding year's effect on isolated corn root tips, 4 herbicides gave more pronounced alterations than the preceding year, and 3 herbicides were more or less identical in their action for the second year as the first year. Since the plant material and the mode of herbicide application are different between the two year's study, it is difficult to ascribe these differences and similarities to specific cause, but these responses may represent species specific responses. Herbicidal effects were not detected not only in the leaf cells but in shoot meristematic cells. Since herbicides were not forced in this year, the difference in observations between two years should indicate that the accumulation of herbicides was less in the shoot this year than the preceding year.

Task 3: Examination of Organelles in Treated Tissues

Attempts were made to isolate and purify mitochondria from herbicide treated soybean root tips and chloroplasts from treated soybean leaves. Purification of mitochondria was attempted with a density gradient centrifugation method; it appears that bromacil treatment tends to change the bouyant density of mitochondria, but not DNOC treatment. Chloroplasts isolated by means of differential centrifugation showed less activity of photosystem II from herbicide treated leaves than control. Because of sizable experimental variations, these observed differences need further confirmation.

Diallate appears to make isolated mitochondria aggregate if the fixative (2% glutaraldehyde) was added to the mitochondrial pellet after diallate treatment, but not if the fixative was added to the mitochondrial suspension to which diallate had been given. These curious observations may suggest that diallate alters the properties of outer mitochondrial membrane.

D. OVERALL EVALUATION OF METHOD

Based on our initial idea summarized in Section B and Fig. I-1, a recording instrument was developed to anlyze simultaneously leaf photosynthesis, transpiration, and root respiration using an intact soybean plant. Some aspects of recording were automated. A herbicide was given to the root solution of the plant. The capability of this instrument can be summarized as follows:

a. It records the three physiological processes of the plant for about 8 hours continuously without much change in rates,

b. It detects the speed of herbicidal action on the three processes in temporal sequence,

c. From the sequence of herbicidal effects, the mode of action of herbicidal phytotoxicity can be deduced,

d. If a herbicide does not affect the three processes in 6 hours at high concentrations, it is likely that the herbicide is either not taken up by the root or acting on other physiological processes.

It should be emphasized that there is a high degree of correlations between the effects observed in the instrument and the effects observed by biochemical means used in the first year. For example, if leaf photosynthesis is inhibited much before transpiration is inhibited, the herbicide which causes such an effect is a photosynthetic inhibitor as detected in the first year in our chloroplast assay. Such an inhibitor usually does not affect root respiration. DNOC affected both mitochondrial respiration and chloroplast photosynthesis in our *in vitro* assays of the first year; this herbicide, when applied to the root solution, it inhibits root respiration first and then leaf photosynthesis in this instrument. Amitrole whose action was not detected in biochemical analyses of the first year acts to inhibit both leaf photosynthesis and transpiration simultaneously with this instrument, although the inhibition appears slowly. Simultaneous inhibition of leaf photosynthesis and transpiration strongly suggests that this herbicide has an ability to inhibit the mechanism of stomatal opening.

We strongly feel that this instrument has a good potential to be used for inital screening of herbicidal phytotoxicity. For herbicides whose activities are detected by this instrument, one can make a good assessment of biochemical modes of action of the herbicides, in view of good correlations between the instrumental detection of the effects and the biochemical assessment of the effects. For slowly or not acting herbicides as detected by this instrument, one can direct attention to other physiological and metabolic processes. Trifluralin belongs to this category.

The new, rapid method of Muller and Jacks facilitates faster assessment of herbicidal effects on alterations of ultrastructure. The examination of ultrastructural changes continues to be highly helpful, particularly for those herbicides which (a) alter overall cellular structures (e.g., 2,4-D), (b) affect primarily the mitotic activities of meristematic cells (e.g., trifluralin) which cannot be detected by the combined instrumentation, and (c) inhibit the three physiological processes very slowly. As an additional method, the analysis of ultrastructural alterations must be included in the screening of herbicidal phytotoxicity.

The analysis of isolated organelles, either from untreated or herbicide treated tissues, by means of conventional biochemical methods may not necessarily provide information concerning the primary mode of action of herbicides in the metabolism of intact plants. In general, the same herbicide inhibits in vitro assays of different organelles at a comparable or different range of concentrations. On the other hand, such assays may reveal additional information as to the action of herbicides. We recommend that in vitro assays with isolated organelles be performed when (a) the instrument such as the one developed cannot provide definitive information, and the more exact biochemical mode of phytotoxicity must be investigated.

As stated before, we consider that the instrument developed in this project has a good potential to be used for screening of phytotoxicity. The instrument we used is still a developmental model. If this type of instrument is to be used in actual screening, we recommend further improvements; e.g., (a) to improve the trap system for longer usage of the instrument, (b) to change the manual aeration of the root solution to automatic aeration so as to save manual labor (electrolytic generation of oxygen can be connected to the oxygen electrode for measurement of root respiration), and (c) to automate the data processing system by connecting to a computer so as to obtain the rates of root respiration and leaf photosynthesis directly from the instrument, instead of manually calculating from the slopes of recorder traces.

CHAPTER II

DEVELOPMENT OF SIMULTANEOUS MEASUREMENTS OF SHOOT PROCESSES

By Howard H. Robinson, Charles F. Yocum, and Hiroshi Ikuma

INTRODUCTION

For maximum photosynthesis to occur in plant leaves, the chloroplasts must be supplied with sufficient water and CO_2. While water is furnished by the root system of the plant, CO_2 must diffuse into the leaves from the surrounding air through the stomata. Under conditions of sufficient water supply, stomatal opening is primarily regulated by light and CO_2 concentration (see Salisbury and Ross, 1969). Light acts to open the stomata, while CO_2 at high concentrations acts to close them even in the presence of light. At low concentrations of CO_2 the stomata are open. Gas exchange through stomata is further influenced by physical, environmental parameters; i.e., leaf temperature, the thickness of diffusion layer outside the stomata, and concentration differences of gas between inside and outside of the leaf (see Salisbury and Ross, 1969; Sestak et al., 1971). For red kidney beans (Phaseolus vulgaris var. Charlevoix) the optimal leaf temperature is about 37° C (Tenhunen et al., 1976). From the Fick's law, the thinner the diffusion layer is, the faster the diffusion becomes, and the larger the concentration difference is, the faster is the rate of diffusion. Empirically, the diffusion layer can be minimized by passing a dry air around the leaves.

Since the degree of stomatal opening can pose a restriction to the diffusion of CO_2 and regulate the photosynthetic activity of mesophyll cells, it is important to examine if a given herbicide affects mesophyll photosynthesis directly or it acts to cause stomatal closure which then indirectly causes a decrease in mesophyll photosynthesis. Thus the analysis of herbicidal effects on mesophyll photosynthesis must be accompanied by measurements of stomatal opening or transpirational activity. We thus attempted to develop an instrument which measures mesophyll photosynthesis and transpirational activity simultaneously.

For our development of instrumentation and establishment of a standardized procedure, we employed young soybean plants. We further tried to maximize mesophyll photosynthesis and to subject a leaf to be used in as natural a condition as it might experience in the field. The following conditions were thus given to the plants for this purpose: (a) a young soybean plant, originally grown in the soil in a planting pot, was transferred to a solution culture condition, (b) a single leaflet of the second trifoliolate leaf was used for measurements, (c) the air of ordinary chemical composition (21% O_2, 79% N_2, and 0.03% (= 300 ppm) CO_2) was passed through the leaf chamber after dehumidification, and (d) the temperature of the leaf chamber and the passing air was kept at 32° C. The condition (a) was made to secure maximum supply of water to the shoot and also to allow easy, uniform application of herbicides to the root. The use of a single leaflet (Condition b) was made after logistic considerations to obtain an accurate assessment of herbicidal effects. The use of low concentration of CO_2 (Condition c) may not give us the maximum photosynthetic capability of the leaflet, but, together with a high temperature (Condition d), the leaflet would provide us with values of photosynthesis and transpiration it would in the field on a dry summer day. Since we provided the leaflet with a high concentration of O_2, it is expected that the measured rates of photosynthesis include photorespiration (see Tolbert, 1973). The effects of herbicides were analyzed with respect to the rates of photosynthesis and transpiration prior to herbicidal addition.

MATERIALS AND METHODS

Before establishment of instrumentation and herbicide screening procedure, a number of modifications were made. In this section the finalized instrumentation and procedure are given. Problems which we encountered during the process of development will be summarized in the RESULTS section.

Instrumentation. Figure II-1 schematically illustrates the instrumentation for simultaneous measurement of photosynthesis and transpiration with soybean leaflet by means of an infra-red CO_2 gas analyzer and a hygrometer, respectively. The middle leaflet of the second fully expanded trifoliolate leaf of a young soybean plant was placed in the leaf sample chamber. The chamber was made of two pieces of lucite cylinder (3¼ inch inside diameter with 1/8 inch wall thickness). One end of the top piece was directly connected to a square lucite heat filter (3 1/8 inch square inside and ½ inch deep) which was connected to a constant temperature circulator (Lauda Thermostat, Type K2-R) set at 32° C. One end of the bottom piece was closed by a sheet of lucite and the other end was jointed to the open end of the upper cylinder and sealed with lanoline. At the juncture of the two cylinders, a small hole was made and the petiolule of the leaflet was placed. After placing the middle leaflet this hole was sealed with lanoline; the rest of the shoot including two leaflets of the second trifoliolate leaf was placed outside of the leaf chamber. The leaflet in the chamber was positioned in the center by means of two coarse screens (one immediately above and the other immediately below the leaflet) made of sewing thread.

The leaflet was placed perpendicular to incident white light from an Oriel tungsten lamp (Model 6325) through the heat filter system. The heat

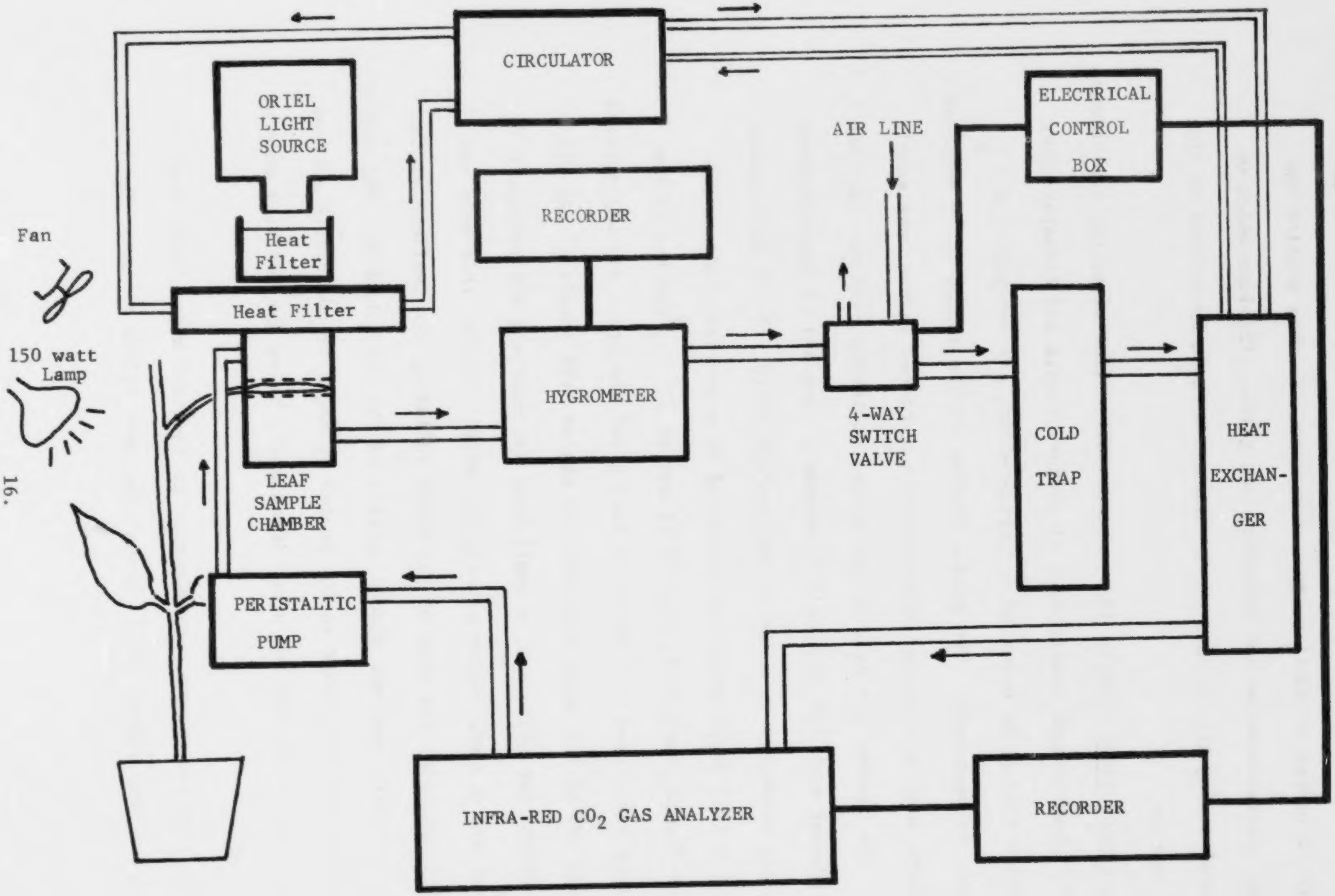

CIRCULATOR
ORIEL LIGHT SOURCE
Heat Filter
Heat Filter
Fan
150 watt Lamp
RECORDER
HYGROMETER
LEAF SAMPLE CHAMBER
AIR LINE
4-WAY SWITCH VALVE
ELECTRICAL CONTROL BOX
COLD TRAP
HEAT EXCHANGER
PERISTALTIC PUMP
INFRA-RED CO2 GAS ANALYZER
RECORDER

Fig. II-1. Diagramatic presentation of the instrument for simultaneous measurements of photosynthesis and transpiration. See text for details.

filter system was composed of two parts: a 600 ml beaker filled with distilled water (10 cm deep) and a closed square lucite filter described above.

The gas line from the sample chamber (from the lower cylinder) was connected to a thermoelectric dew point hygrometer (Cambridge Systems Inc., Model 880) to monitor water vapor concentrations in the outlet gas from the leaf chamber. The hygrometer reading was recorded on a Heath-Schlumberger recorder (SR-255B).

The gas line was then connected to a cold trap via a 4-way solenoid switch valve. The 4-way solenoid valve between the hygrometer and the cold trap allowed the whole gas circulation system to be closed or open alternately; i.e., the valve made connections between the hygrometer and the cold trap for a closed mode to measure mesophyll photosynthesis and transpiration, or dissociated the connection so as to have the leaflet in the chamber exposed to the external air between the periods of measurements. Since the CO_2 concentration in the laboratory changed depending on the presence of people, an outlet from the building air line was connected to the solenoid valve after passing through a large volume of water. The alternate switching between the open and the closed modes allowed the leaflet in the sample chamber to maintain a constant physiological condition for at least 8 hours.

The cold trap was made of two copper tubings each 2 feet long; one was a tubing of 3/4 inch inside diameter (1/16 inch wall thickness) with an air-tight tubing cap on the bottom end and the other was a tubing of ¼ inch inside diameter (1/32 inch wall thickness). The narrower tubing was inserted into the larger tubing, with the inner tubing protruding about 10 cm beyond the upper edge of the outer tubing. An air-tight copper pipe coupler held the two tubings in position. The inlet gas from the solenoid valve entered into the outer, wider tubing and exited from the inner, narrower tubing. The entire cold trap was held upright and about 40 cm of the bottom end was inserted into a dry ice bath (dry ice in alcohol, -56° C). The cold trap was inserted in the gas circuit to remove the moisture from the gas line before monitoring CO_2 concentrations by the gas analyzer; water vapor interferes with CO_2 measurements by the gas analyzer.

The heat exchanger was composed of spiral copper tubing (¼ inch diameter) inserted into a water bath at 32° C. The water bath was made of a pyrex glass tubing (2½ inch inside diameter and 6½ inch long) which was connected to a Lauda constant temperature circulator set at 32° C. The purpose of the heat exchanger was to raise the temperature of the gas before it entered the gas analyzer (Beckman gas analyzer, Model 864). A recorder (Heath-Schlumberger recorder, Model SR-255β) was connected to the gas analyzer to record CO_2 concentrations.

A peristaltic pump (Cole-Parmer Masterflex tubing pump) allowed the unidirectional circulation of the gas, as indicated by arrows in Fig. II-1. The gas was circulated at a rate of 800 ml/minute.

The electric control box housed an electrical device which allowed alternate switching of the solenoid valves to open and close and simultaneously regulated the chart drive of the recorder connected to the gas anlyzer (Fig. II-2 for the schematic of this control circuit). In an open mode of the solenoid switch, the current was removed from the solenoid valve to make them relaxed, the chart drive of the recorder was turned off, and the recorder pen was lifted. In the closed mode of the switch, the current energized the solenoid valve to route the gas from the hygrometer to the cold trap, the recorder chart drive was activated, and the recorder pen was down to record CO_2 concentrations as monitored by the gas analyzer. This electric device gave timed alternations of open and closed modes of the solenoid switch.

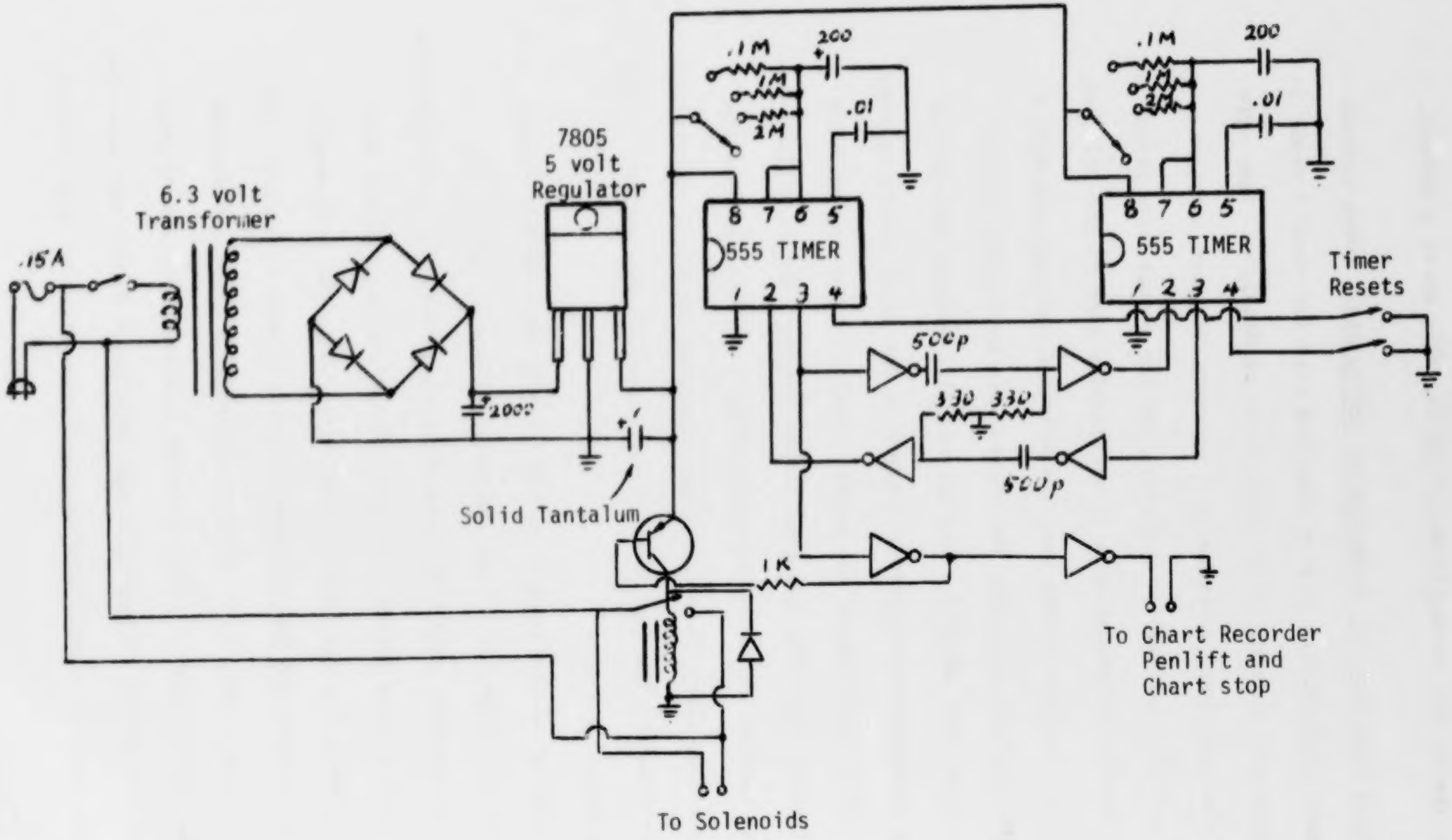

.15A
6.3 volt
Transformer
.2000
7805
5 volt
Regulator
Solid Tantalum
1
.1M
1M
2M
200
.01
8 7 6 5
555 TIMER
1 2 3 4
.1M
1M
2M
200
.01
8 7 6 5
555 TIMER
1 2 3 4
Timer
Resets
500p
330 330
500p
1K
To Chart Recorder
Penlift and
Chart stop
To Solenoids

Fig. II-2. Schematic diagram of the circuit in the electronic circuit box.

Resistors are in OHMS; capacitors are in MFD; diodes are 1N 4004; the transistor is PNP 2N 3906; relay is Calectro No. D1 966 6V DC coil; -▷o are 1/6 of a 7404 4EX inverter.

In our routine tests, the automatic switching was done every 3 minutes.

Standardized Procedure for Screening of Herbicides. Young soybean plants were grown individually in clay planting pots for about 8 weeks in the Matthaei Botanical Gardens of the University of Michigan. They were de-soiled and the roots were immersed in ½ strength Hoagland nutrient solution, the solution was bubbled with air, and the plants were placed in the greenhouse for an additional week to acclimatize them to the solution culture condition. A night before use, the solution was replaced with a fresh ½ strength Hoagland solution, the plants were kept under diffuse light, and the roots were aerated. Wet air was also bubbled through the solution during measurements to provide oxygen to the roots and to agitate the solution. After insertion of the middle leaflet of the second fully expanded trifoliolate leaf into the leaf sample chamber, the Oriel lamp was turned on to the leaflet and a 150 watt photoflood lamp was given to the rest of the plant from about 60 cm distance. In order to avoid temperature rise during measurements, the entire plant shoot, except the leaflet in the chamber, was agitated by a fan.

Since the stomata of the leaflet in the chamber were usually closed immediately after the insertion of the leaflet, the entire set-up was run for a period of 60 minutes, until the rates of transpiration and photosynthesis became steady. During this period, the gas circuit was held in the open mode; i.e., atmospheric CO_2 was continuously passed into the leaf sample. Once the stomatal opening and photosynthetic activity were stabilized, the electrical control was turned on to provide alternating cycles, 3 minutes each, of open and closed modes. After at least 5 complete cycles of open and closed modes, a solution of herbicide was introduced to the root solution and the herbicidal effects on photosynthesis and transpiration were continuously monitored for 6 hours or until the rate of CO_2 uptake becomes zero. The time course of herbicidal effects was examined and the effects were compared against the same leaflet before herbicide addition.

Plants with practically identical growth were selected for each set of herbicide treatment. The plants were at the stage when the second trifoliolate leaf completed its full expansion and the third trifoliolate leaf was still developing.

RESULTS

A. DEVELOPMENT OF THE STANDARD PROCEDURE

In order to analyze the modes of action of herbicides using the shoot of an intact plant, we decided from the beginning not to use the entire shoot of a soybean plant. This decision was based on foreseeable technical difficulties; for example, the plant chamber must be large to house the entire shoot, high intensity light must be given uniformly without heating the shoot excessively, and the air must be passed through the sample chamber rapidly and uniformly. Obviously, the shoot in the chamber must experience a strong wind during the period of measurement to the extent that some leaves might be broken. With the use of a single leaflet, the sample can be held practically motionless, high intensity light can be given uniformly throughout the leaflet surface, and the construction of the sample chamber is more manageable.

Even with the use of a single leaflet, various factors which affect its photosynthesis and transpiration must be kept constant at an optimal condition. The physiology of the leaflet in the chamber must not be affected by the rest of the plant during measurements and the instrument must function properly throughout the measurement period. This section summarizes several problems which arose during the preparatory phase of this work.

Development of Instrumentation. The entire set-up consists of the leaf sample chamber, a hygrometer, the dehumidifying system (a cold trap and a heat exchanger), and an infra-red CO_2 gas analyzer, and an air circulation pump (see Fig. II-1). We endeavored to make a set of automated recording devices. For this purpose, a 4-way solenoid valve system was inserted between the hygrometer and the dehumidifying system, because solenoid valves could be electrically timed and controlled. In the circuit of this controlling device, we added a circuit to regulate the operation of the recorder for the gas analyzer to save unnecessary usage of the chart paper.

Because both CO_2 and water vapor absorb infra-red light, the input air to the gas analyzer must be free of water vapor. An insertion of dehumidifying system thus became necessary. This necessarily made the circulation of dry air into the leaf chamber, which helped to monitor small changes in water vapor more accurately. The position of the solenoid valve was thus fixed between the hygrometer and the dehumidifying system, instead of between the gas analyzer and the leaf chamber. Furthermore, with the solenoid valve in this position, the transpirational activity of the leaflet was kept unchanged between the open and the closed modes of solenoid valve.

All necessary connections of gas lines were made with flexible amber gum rubber tubing. This allowed the entire system at equilibrium with the external air pressure, except the period when abrupt changes of barometric pressure occurred such as a passage of a thunder storm. It should be noted that practically all tubing materials can pass CO_2 across the wall. We used amber gum rubber tubing of relatively thick walled variety, instead of tygon tubing, mainly because of flexibility. A barometric pressure higher than the surrounding air is given in the gas circulating system by means of a peristaltic pump.

As indicated in the introduction to this chapter, we employed ordinary air, instead of a high CO_2 mixture, mainly because the plants in the field experience the 300 ppm concentration of CO_2. A CO_2 concentration of 1500 ppm may maximize mesophyll photosynthetic activity, but the results may be unnatural in the field. The temperature of the circulating gas to the leaf chamber was adjusted to 32° C, because (a) temperature alterations could be avoided from our set-up (the temperature of the output air from the gas analyzer was about 32° C), and (b) this temperature was considered to be close to the summer leaf temperature in the field.

Conditioning of the Plant. When young soybean plants, either in planting pots or in solution culture were brought into the laboratory from the greenhouse and immediately subjected to measurements of photosynthesis and transpiration, they responded in an unexpected manner: for example, the stomata did not close properly in the dark nor open properly in light. In the dark, the hygrometer tracing drifted slowly towards lower concentration of water vapor, and in light water vapor concentrations oscillated for 4-6 hours after the Oriel lamp was turned on. The degree or the oscillation of stomatal opening obviously affected the respiratory and photosynthetic activities of the leaflet. Illumination of the rest of the plant with diffuse light somewhat reduced the amplitude of oscillation, but the oscillation continued for a long time. A use of a plant which was dark adapted for one day showed a steady low level of stomatal opening in the dark, but the long oscillation of stomatal opening was not removed in light. Plants adapted for a long time in bright light or in the dark were thus proved to be unuseable to observe subtle effects caused by herbicides, unless the herbicide treatment was delayed about 8 hours after setting the plant in the instrument.

Since the measuring system was designed to utilize a single leaflet, the physiology of the leaflet would be affected by the rest of the shoot which was placed in diffuse light. After a number of systematic trials, we adopted the following preconditioning scheme: (a) de-soiled plants were adjusted to the water culture condition for about a week, and (b) a goose-neck lamp with a 75 watt tungsten lamp was placed about 60-80 cm away from the plants for overnight. During the adjustment period in solution culture, the root tips which had been damaged by the de-soiling procedure recovered and new roots started to develop. The preconditioning of the plants under diffuse light allowed the plants to respond to treatment in an expected manner (see next section).

B. PERFORMANCE OF INSTRUMENT

Figure II-3 illustrates a typical set of recorder tracings from the gas analyzer and the hygrometer by our standard procedure. The middle leaflet of the second trifoliolate leaf was placed in the leaf sample chamber shortly before the start of tracing and the entire plant was kept in the dark. The Oriel lamp, the supplementary 150 watt photoflood lamp, and the fan were turned on simultaneously at the time of the LIGHT ON arrow. The CO_2 concentration was monitored for 3 minutes with the solenoid valve in the closed mode. The recorder was not run between recordings, when the gas circuit was in the open mode for 3 minutes. The hygrometer water vapor trace was taken continuously throught the measurement period.

In all recorder tracings of the gas analyzer, it takes about 20 seconds before the CO_2 level starts decreasing, the CO_2 level abruptly decreases thereafter before reaching another steady level for about 20 seconds, and the rate of CO_2 uptake becomes linear. We interpret these observations that it takes about 20 seconds for complete cycle of the gas through the

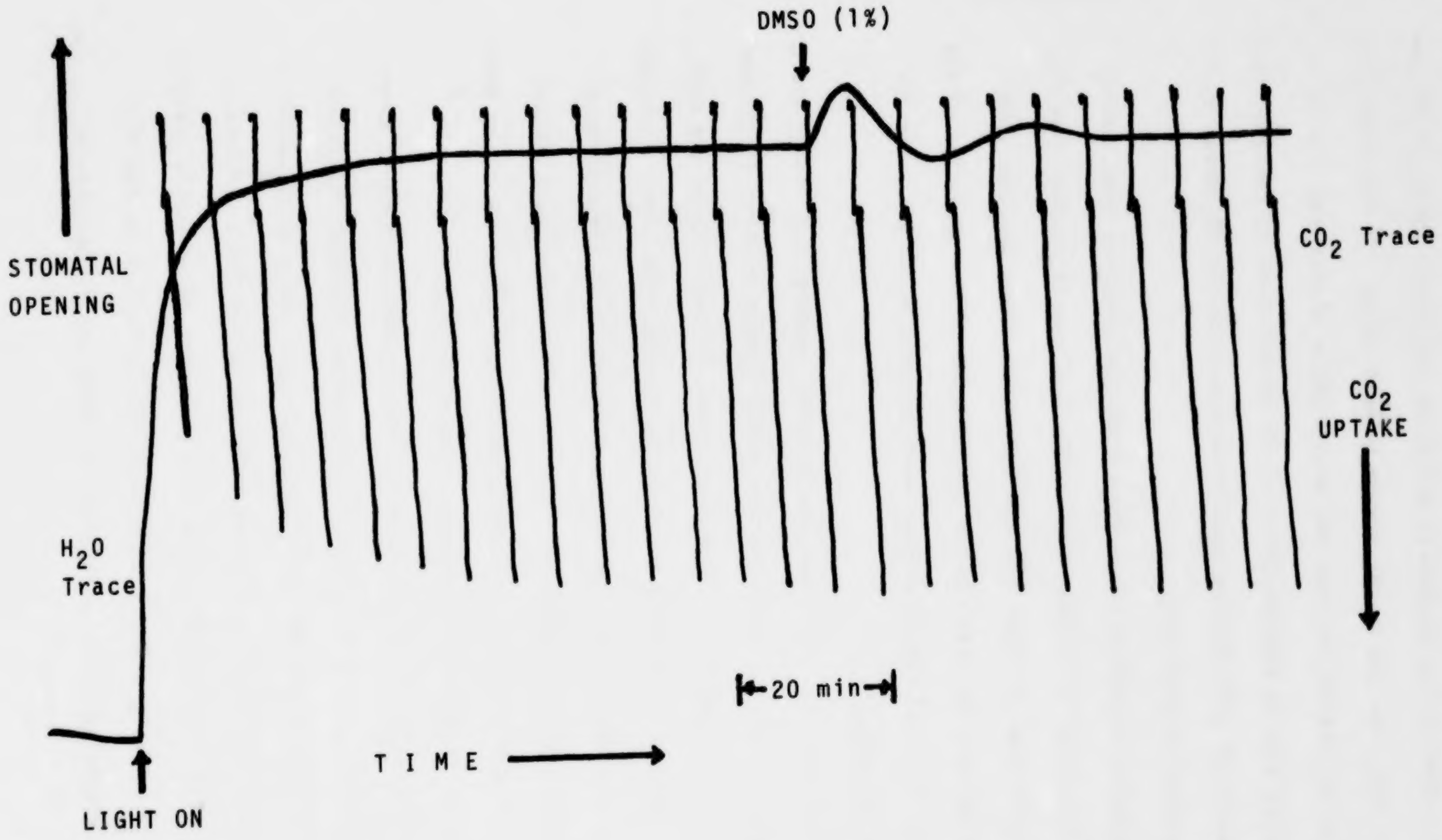


Fig. II-3. A typical set of recorder tracings from the hygrometer and the infrared CO_2 gas analyzer.

The CO_2 traces were taken every 3 minutes for 3 minutes, indicating the photosynthetic activity of the leaflet. The hygrometer (H_2O) trace indicates the degree of stomatal opening. 1% DMSO added to the root solution as indicated.

the gas line system in the instrument and after the second cycle of the gas in the closed mode, the gas in the system is well mixed. In our calculation of the rate of CO_2 uptake, we used the second phase of decrease. It is of interest to note that an extrapolation of the second phase of decrease back towards the start of each tracing usually matches with the CO_2 level at the onset of recording of each trace.

The hygrometer recording trace shows a shorter response time, mainly because the hygrometer is located next to the leaf sample chamber (see Fig. II-1). It should also be noted that the hygrometer trace does not show discontinuity between the open and the closed modes of the operation of the solenoid valve; the leaf sample chamber must be given equally dry air for both modes of the valve.

It is clear from Fig. II-3 that the stomatal opening (H_2O trace) and the rate of CO_2 uptake approach to respective steady levels in about 30 minutes after the light is turned on. Although not shown in this figure, the same steady levels continue up to 8 hours after the initial rise. The limitation of this set-up in terms of the length of measurement is in the cold trap: i.e., if the cold trap is clogged with ice, the measurements give erratic tracings. In our routine test, the measurements were terminated after about 6 hours or earlier if herbicidal effects appeared earlier.

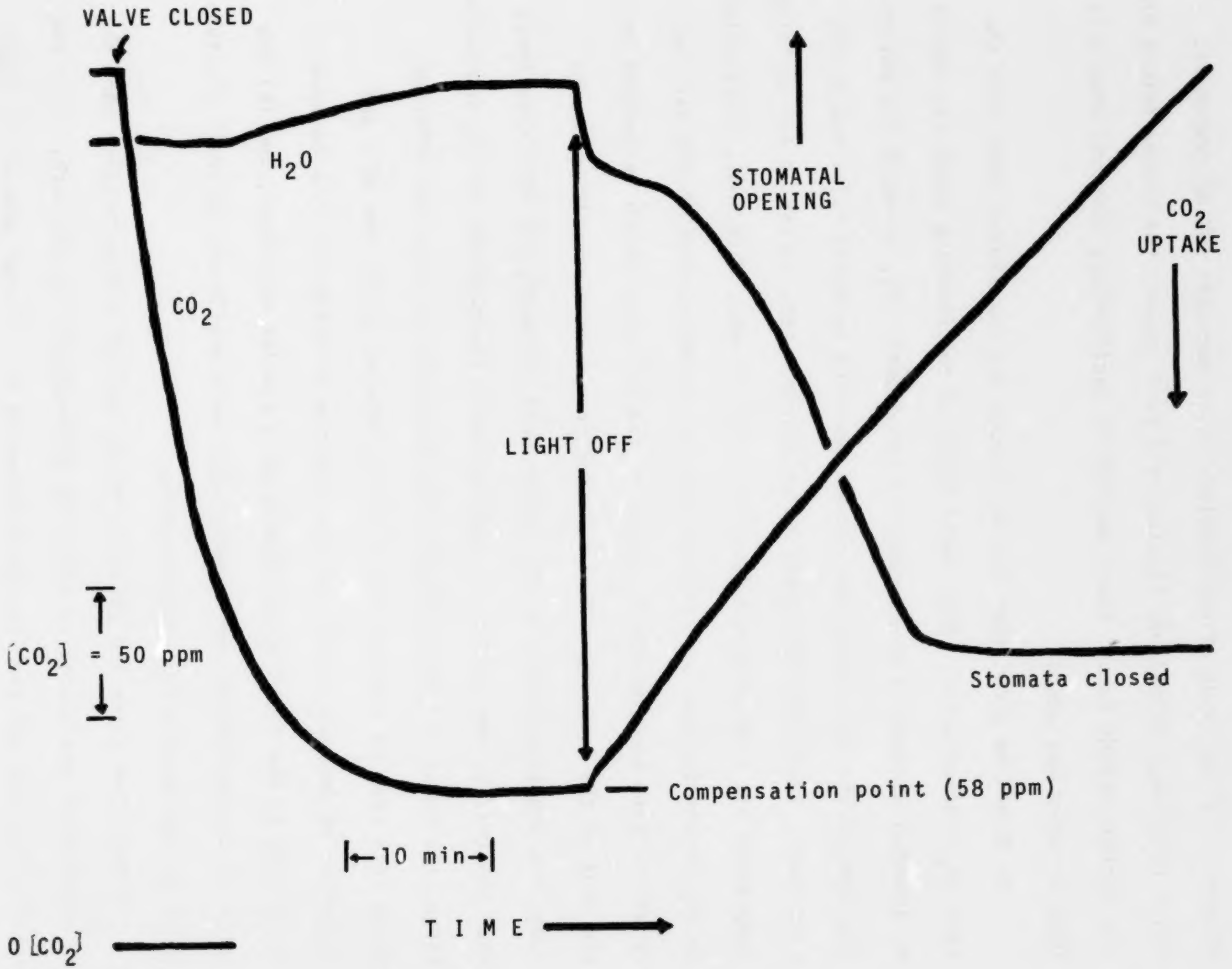
Figure II-3 also shows an effect of 1% DMSO (dimethylsulfoxide) given to the root solution. Stock solutions of many water-insoluble herbicides were prepared in DMSO at 0.1 or 0.01 M concentration, and a small amount of the stock solution was added to the root solution to the final concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M. Since this procedure of herbicide addition necessitates simultaneous addition of DMSO at 10, 1, or 0.1% to the root solution, this figure serves as a control for those water-insoluble herbicides.

As indicated in the figure, DMSO addition at 1% or 10% frequently, but not always, caused a small oscillations in both tracings of water vapor and CO_2 uptake. The amplitude of oscillations never exceeded 10% of responses. After temporary, damped oscillation of 1 to 2 cycles, the traces return to the earlier steady rate. There was little oscillations observed when 0.1% DMSO or water was added.

It should be added that the CO_2 traces did not always start from the same CO_2 concentration. This small drift of the starting point was caused by somewhat altered concentrations of atmospheric CO_2, to which the entire gas line of the instrument was exposed when the solenoid valve was in the open mode. Since the instrument is connected to the outlet of the building compressed air line when the solenoid valve is open, this small fluctuation in CO_2 concentrations must reflect the CO_2 concentrations in the air when it enters into the compressor system of the building which is located on the roof of the building. When the traffic around the building became busy, the starting points of CO_2 traces were elevated, and they were lower when the traffic was not busy. Despite small fluctuations in CO_2 concentrations, the rates of photosynthesis and transpiration were not affected. While this type of fluctuations could be reduced by the use of a gas cylinder, we decided to use the air from the building air line because (a) plants in the field would experience a similar situation, and (b) the rates of photosynthesis and transpiration were unaffected by small fluctuations in atmospheric CO_2 concentrations.

Figure II-4 shows the patterns of CO_2 and H_2O concentrations when the solenoid valve was closed in light and subsequently in the dark. When the valve was closed and the instrument operated in a closed mode while light was on, the CO_2 concentration in the gas line decreases exponentially to a

Fig. II-4. Examination of the performance of the instrument by closing the solenoid valve and turning light off.



steady low level. This level must represent the apparent compensation point where the rate of CO_2 consumption equals the rate of CO_2 evolution. Since the O_2 concentration in the leaf chamber is high, the CO_2 evolution rate must represent the sum of mitochondrial respiration and photorespiration. The water vapor trace shows an increase after the CO_2 concentration is lowered below about 150 ppm. Since the stomata are expected to open at low CO_2 concentrations and close at high concentrations, the increase in H_2O level is expected. When light was turned off after CO_2 and H_2O had reached to new steady values, the stomata close in response to darkness and CO_2 levels increase due to dark mitochondrial respiration. These results thus indicate that the instrument works in an expected manner.

In the field of photosynthesis, it is customary to express photosynthetic rates on the basis of unit chlorophyll content. In our examination of the rate on this basis (Table II-1), we obtained an average rate of 1.28 µmoles CO_2 fixed/mg chlorophyll·minute or 76.8 µmoles CO_2 fixed/ mg chlorophyll·hour with an error range of about 20%. This value is comparable to the basal O_2 evolution value of 50-100 µmoles/mg chlorophyll·hour ($H_2O \longrightarrow$ ferricyanide) in isolated soybean chloroplasts (see the Final Report of the preceding year).

If the light intensity is not saturating, a possible problem may be caused by flapping of the leaflet in the leaf chamber and/or by the use of leaflet with varying degrees of greenness. We have thus tested the effect of light intensity on CO_2 uptake. A typical set of these experiments is summarized in Table II-2, where the same leaflet was used for all measurements. The results indicate that our standard light intensity (117 kergs/cm^2·second) is well in the saturating dosage.

Table II-1

The rate of CO_2 fixation by soybean leaflet.

After measurements of CO_2 fixation by our standard procedure, the chlorophyll in each leaflet used was extracted with 80% acetone, and its content was measured by the method of Arnon. The rate of CO_2 uptake was then calculated on the basis of mg chlorophyll.

Plant number	Observed rate of CO_2 fixation	Chlorophyll in the leaf used	Specific rate
	(μmoles/leaf·min)	(μgm)	(μmoles/mg Chl·min)
1	0.43	410.2	1.05
2	0.43	351.4	1.22
3	0.49	310.6	1.56
Average specific rate			1.28

Table II-2

Effect of light intensities on the rate of CO_2 fixation.

The rate of CO_2 fixation was determined with the same leaflet at three different light intensities. The standard light intensity was 1.17×10^5 ergs/cm^2·sec. Reduced intensities were obtained by inserting metal screens between the light source and the heat filter.

Light intensity		Rate of CO_2 fixation	
Measured value	% of standard light	Observed rate	% of standard condition
(ergs/cm^2.sec)	(%)	(Relative)	(%)
1.17×10^5	100	60	100
0.88×10^5	75	59	98
0.70×10^5	60	49	82

C. EFFECTS OF HERBICIDES ON PHOTOSYNTHESIS AND TRANSPIRATION

Figure II-5 illustrates a way by which herbicidal effects on photosynthesis and transpiration were examined using 10^{-3} M atrazine addition as an example. A herbicide was added to the root solution after a steady rate of CO_2 uptake and a steady degree of stomatal opening were established. The rate of photosynthesis was calculated from each trace of CO_2 uptake: the rates after herbicide addition were compared with the steady control rate before herbicide, with the control rate being 100%. The degree of stomatal opening was calculated as follows: the water vapor concentration before herbicide addition was 100% and the steady low level in the dark before the Oriel lamp was turned on was 0%.

Figures II-6 - II-8 show the effects of atrazine, bromacil, and amitrole on the two leaf processes at 10^{-3}, 10^{-4}, and/or 10^{-5} M. Atrazine and bromacil are relatively insoluble in water, and DMSO was used to solubilize them, while a water-soluble herbicide, amitrole, was dissolved in water. DMSO concentrations of 10% (for 10^{-3} M), 1% (for 10^{-4} M), and 0.1% (for 10^{-5} M) were used. For all three figures, dotted lines are the hygrometer recorder tracings for transpiration and continuous lines with data points represent the rate of CO_2 uptake or photosynthetic rate. Although 10^{-5} M amitrole was examined, there was no change in the rate of photosynthesis and in the degree of stomatal opening for 8 hours: the results are thus not presented.

Atrazine and bromacil are photosystem II inhibitors in isolated chloroplasts (see Final Report of the preceding year). They inhibit both photosynthesis and transpiration: the higher the concentration was used, the faster was the inhibition (Figs. II-6 and II-7). It is noteworthy that these herbicides inhibited CO_2 fixation before transpiration, particularly at higher concentrations. Also, the degree of inhibition of transpiration

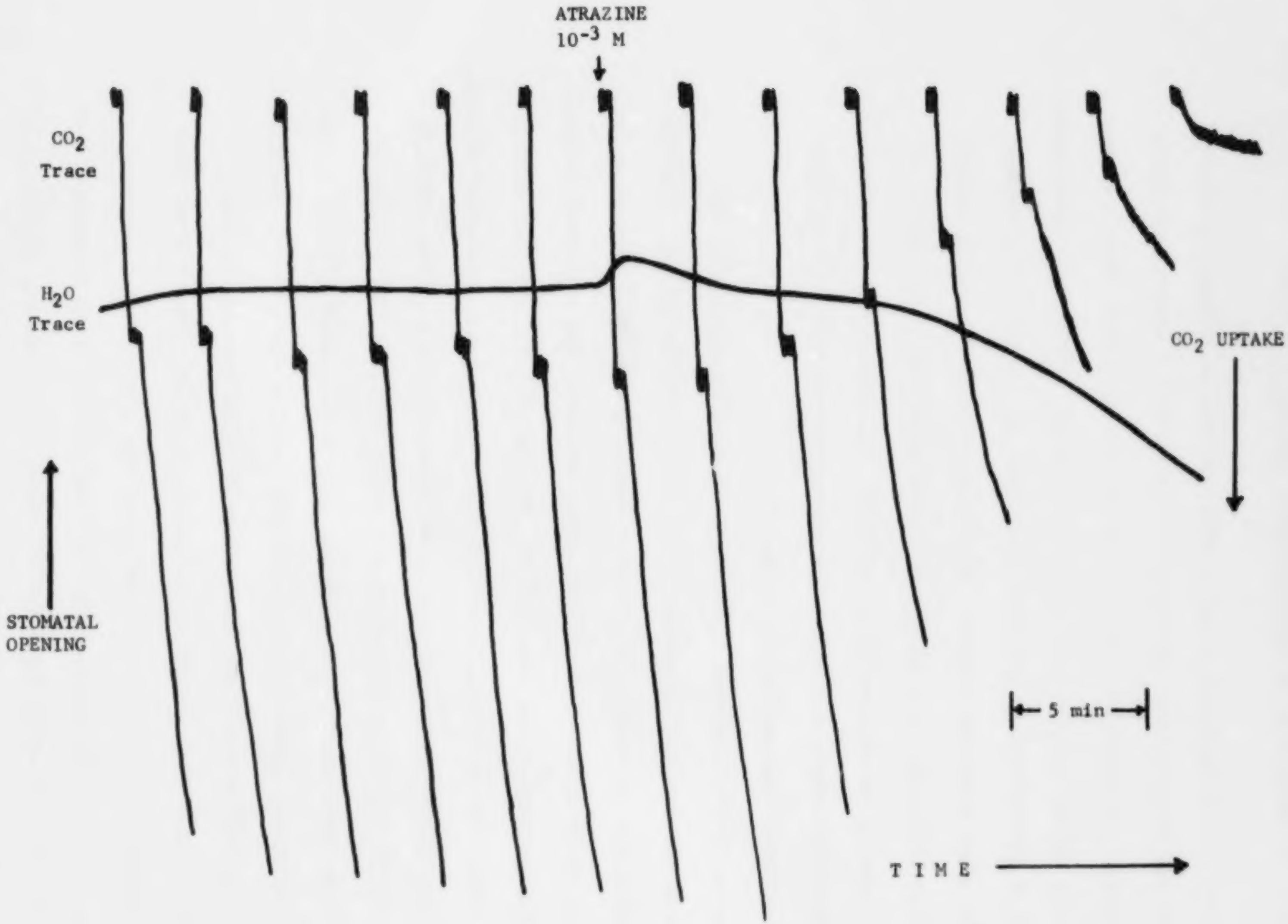


Fig. II-5. A typical set of recorder tracings showing the effects of atrazine at 10^{-3} M on photosynthesis and transpiration.

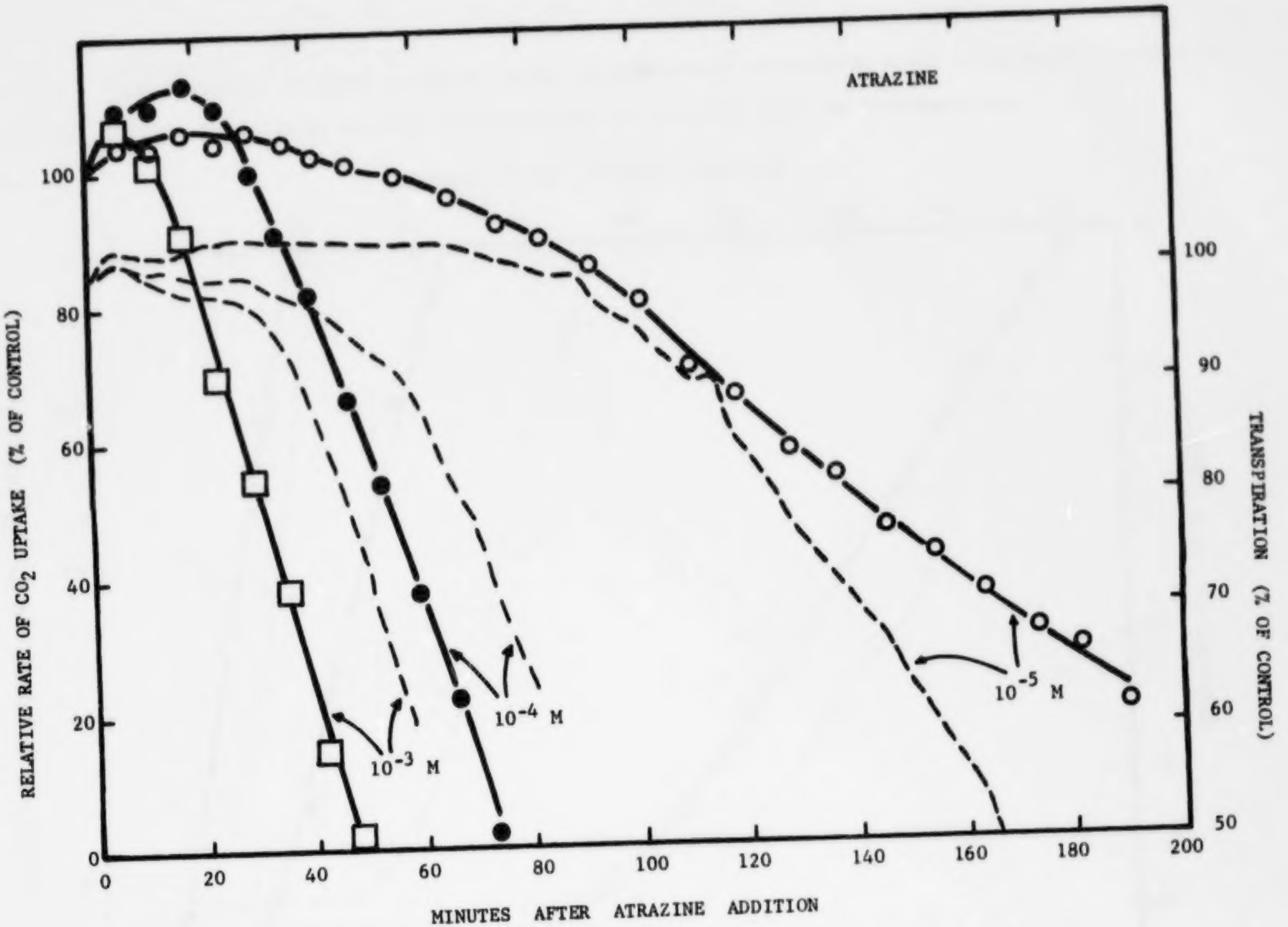


Fig. II-6. Effect of atrazine at three concentrations on photosynthesis and transpiration.

Transpiration is shown in dotted lines; photosynthetic activity is shown in solid lines with data points.

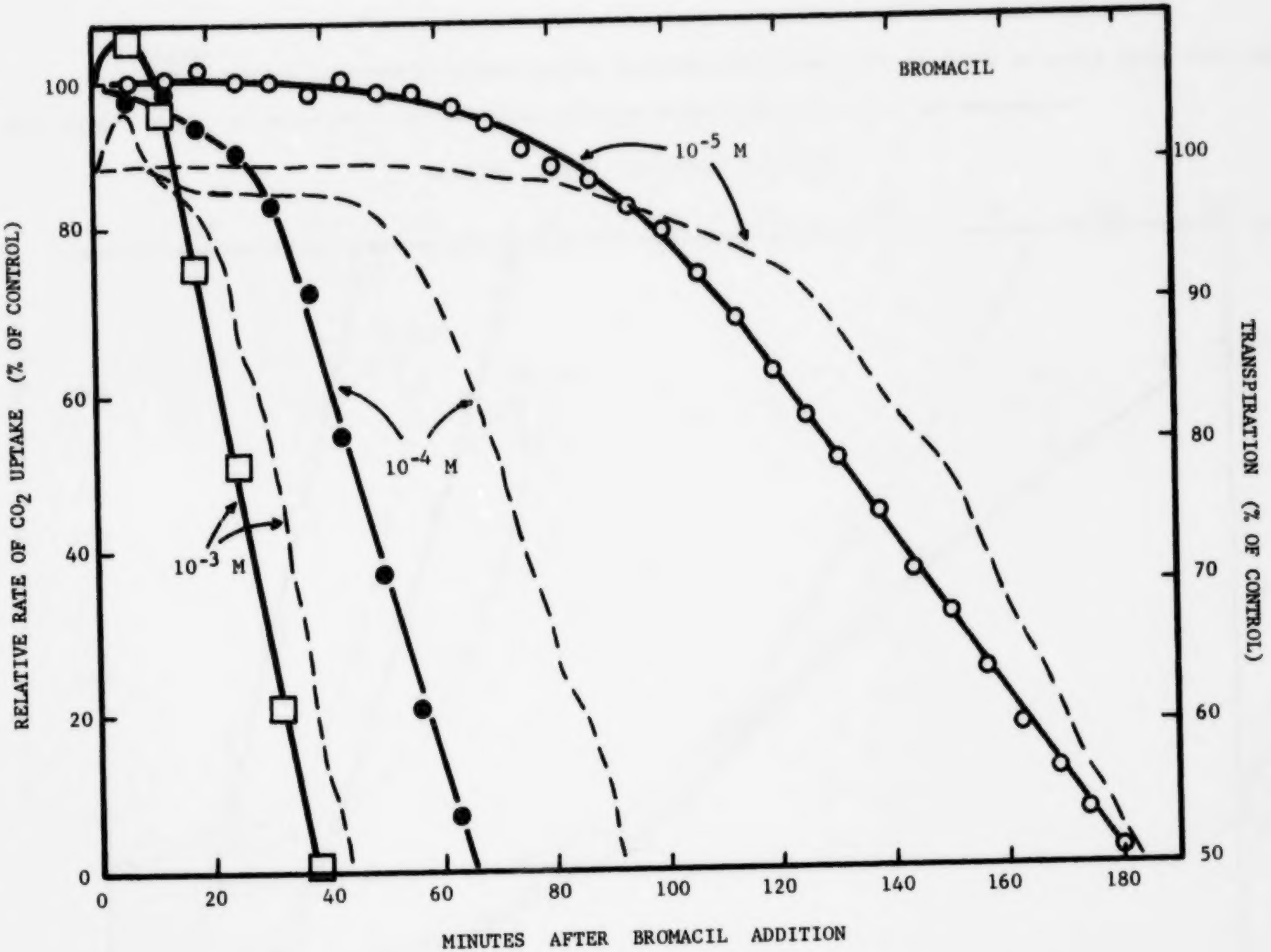


Fig. II-7. Effect of bromacil at three concentrations on photosynthesis and transpiration.

Transpiration is shown in dotted lines; photosynthetic activity is shown in solid lines with data points.

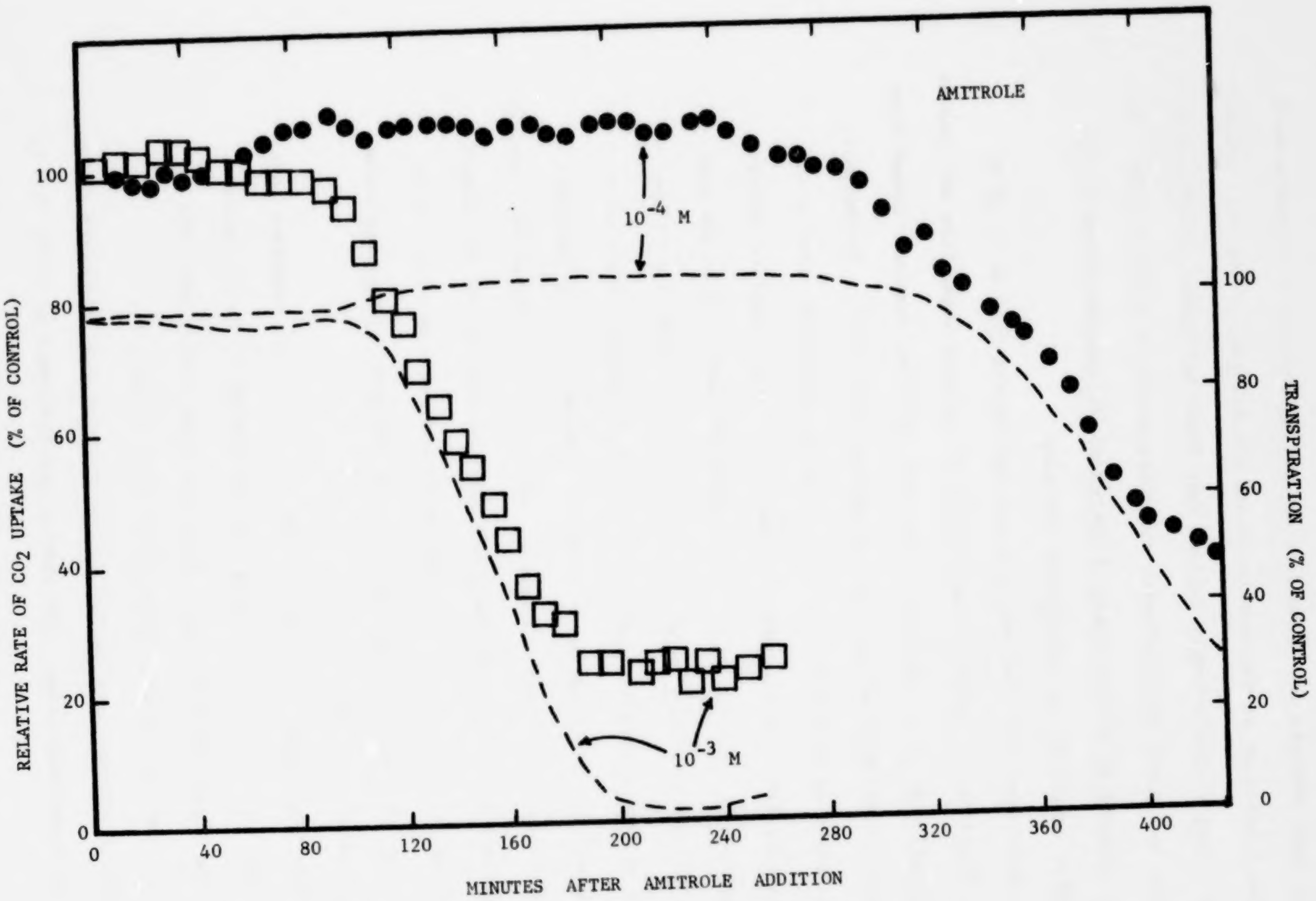


Fig. II-8. Effect of amitrole at two concentrations on photosynthesis and transpiration. Transpiration is shown in dotted lines, photosynthetic activity is shown with data points.

was less (see ordinate for transpiration) during the period of measurement. The quick inhibition of photosynthesis and the delayed, incomplete, inhibition of transpiration strongly suggest that these herbicides act primarily to inhibit mesophyll photosynthesis and subsequently to close stomata. The stomatal closure is likely caused by increased CO_2 concentrations in the substomatal cavity due to respiratory activity.

We noted last year that amitrole was ineffective even at 10 mM in causing inhibition of metabolic activities of isolated organelles and tissues. The application of this herbicide to the root solution, however, caused slow but definite inhibition of both leaf processes (Fig. II-8). Furthermore, the time course of inhibition indicates that the inhibition sets in at about the same time and progresses in parallel. Also, complete closure of stomata can be attained at 10^{-3} M at about the same time as the near complete inhibition of CO_2 uptake. Since stomatal closure limits the supply of CO_2 to mesophyll chloroplasts, it is anticipated that, if a herbicide were to act primarily on stomatal closure, it would inhibit mesophyll photosynthesis simultaneously. We would like, therefore, conclude that amitrole directly acts on the guard cells of stomata and its effect on CO_2 uptake is a secondarily and indirectly induced phenomenon, in a manner similar to the effect of abscisic acid and *Helminthosporium* pathotoxin (Arntzen et al., 1973).

The fact that these herbicides caused any inhibition indicates that they are taken up by the soybean root and transported up to the leaflet. Obviously the higher the concentration is used, the faster would the uptake by the root be and the faster would their effects be.

Herbicidal effects were further tested with the same procedure as the above for 12 other herbicides. The results are tabulated in Table II-3 in

Table II-3

Time required for 50% inhibition of photosynthesis and transpiration of soybean leaflet by herbicides

HERBICIDE	Conc. used (M)	TIME FOR 50% INHIBITION		REMARKS
		Photosynthesis	Transpiration	
		(minutes)		
Amitrole	10^{-3}	145	155	
	10^{-4}	390	400	
Atrazine	10^{-3}	30	70	
	10^{-4}	55	95	
	10^{-5}	140	170	
Bromacil	10^{-3}	28	44	
	10^{-4}	50	92	
	10^{-5}	130	185	
Cacodylate	10^{-2}	170	295	
Chlorpropham	10^{-3}	115	200	
	10^{-4}	(270)	(420)	
2,4-D	10^{-3}	300	320	
Diallate	10^{-3}	160	>240	Incomplete inhibition of transpiration (45%) in 4 hours
Diquat	10^{-3}	95	180	
Diuron	10^{-3}	60	100	Incomplete inhibition at 10^{-5} M in 4 hours
	10^{-4}	130	170	
DNOC	10^{-3}	30	70	
	10^{-4}	105	150	
DSMA	10^{-2}	280	290	Very slight inhibition of photosynthesis at 10^{-3} M in 4 hours
Karbutylate	10^{-3}	15	45	
	10^{-4}	55	68	
	10^{-5}	200	280	
Propham	10^{-3}	190	260	
	10^{-4}	(590)	(590)	
Simazine	10^{-4}	80	160	10^{-3} M not tested because of a solubility problem
	10^{-5}	155	220	
Trifluralin	10^{-4}	---	---	Very slight effect in 4 hours; 10^{-3} M not tested because of insolubility

terms of the time required for 50% inhibition of photosynthesis and transpiration. This table includes the three herbicides already described. It should be added that the concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M were tested for all herbicides and in some cases (cacodylate and DSMA) 10^{-2} M was used, but the table lists only the concentrations which showed some effects in 4-6 hours.

From the survey of the table, it can be concluded that (a) in general, the higher the concentration was used, the faster the effects appeared, (b) within the solubility limit, trifluralin gave practically no effect, and (c) with a lone exception of amitrole, all effective herbicides inhibited photosynthesis much faster than transpiration particularly at higher concentrations, but the difference in effective time between photosynthesis and transpiration became less pronounced as the lower concentrations were used. In terms of the speed of action, the fifteen herbicides can be divided into three groups: quickly acting herbicides, slowly acting ones, and a group of herbicides which act in a intermediate speed. At 10^{-3} M, a group of quickly acting herbicides (atrazine, bromacil, DNOC, karbutylate, and simazine) inhibits photosynthesis within 1 hour (although 10^{-3} M simazine was not tested, this herbicide can be grouped in this category from its effect at 10^{-4} and 10^{-5} M). At the highest concentrations tested, 6 herbicides (amitrole, cacodylate, 2,4-D, diallate, DSMA, and propham) inhibited photosynthesis slowly--50% inhibition appeared more than 2 hours after application. At 10^{-3} M, 3 herbicides (chlorpropham, diquat, and diuron) acted in an intermediate manner on photosynthesis causing 50% inhibition between 1 and 2 hours after application. The speed at which a herbicide inhibits photosynthsis reflects the speed of penetration into the root cells and subsequent translocation of the herbicide into mesophyll chloroplasts.

In comparison with the results of preceding year, the summarized results in Table II-3 indicate striking similarity with those obtained last year with isolated soybean leaf disks (Table V-2 of the Final Report of last year) and even with last year's isolated soybean chloroplasts (Table III-2 of the last year's Final Report). These comparisons strongly suggest that the procedure and instrumentation employed this year can assess herbicidal phytotoxicity accurately. In addition, the procedure employed this year can provide information on stomatal opening, as evidenced by the action of amitrole (Fig. II-8).

DISCUSSION

For proper, simultaneous measurements of photosynthesis and transpiration, the soybean plants must be subjected to a pre-conditioning with diffuse light for several hours. This conditioning of plants was not necessitated by the transfer of the plants to solution culture: the same conditioning was necessary for plants potted in soil. Plants in soil or in solution culture behaved in a similar manner depending upon the time of the day when measurements were taken. Pre-conditoned plants, on the other hand, behaved in a uniform and predictable manner for a long time. Since the measurements were performed on a single leaflet with the rest of the plant in diffuse light, the condition given to the rest of the plant appears to govern the nature of preconditioning of the whole plant.

The leaflet of a pre-conditioned plant behaved in a predicted manner. Stomata opened quickly in light to a near maximal level in dry air (Fig. II-4). A small fluctuations in CO_2 concentrations did not alter either the degree of stomatal opening or photosynthetic activity. The leaflet in the leaf chamber maintained the same physiological condition for a long time. A small perturbation to the root (e.g., DMSO addition) caused a rapid and transient effect, and changes in environmental conditions (e.g., decrease in CO_2 concentrations as in Fig. II-4 and light on or off as in Figs. II-3 and II-4) induced rapid responses in photosynthesis, respiration, and the degree of stomatal opening. Thus, our instrument detects small alterations of the physiological conditions of the plant and the leaflet quite sensitively. It is thus ideal to analyze the mode of action of herbicidal phytotoxicity with the instrumentation and procedure described here.

Our survey of herbicidal phytotoxicity closely resembles the results obtained last year with isolated chloroplasts and soybean leaf disks (Tables III-2 and V-2 of last year's Final Report). Inhibitors of photosystem II of photosynthesis inhibit mesophyll photosynthesis before transpiration. Highly effective herbicides such as atrazine, bromacil, karbutylate, and probably simazine act rapidly in our assay of this year, while they acted at low concentrations in last year's study. Inactive herbicides of last year, such as amitrole, cacodylate, chlorpropham, 2,4-D, propham, and trifluralin inhibit mesophyll photosynthesis slowly in this year's screening. Remaining herbicides acted in an intermediate manner in the studies of both years.

There are several small differences in results between the study of last year and that of this year. For example, amitrole caused very little inhibition of photosynthesis of isolated chloroplasts and leaf disks last year, but it inhibited mesophyll photosynthesis slowly but definitely this year. Diquat was a very poor inhibitor of chloroplast photosynthesis and a relatively strong inhibitor of leaf disk photosynthesis last year, while it inhibited mesophyll photosynthesis in an intermediate manner this year. Diuron or DCMU is a strong, classical inhibitor of photosystem II and it inhibited photosynthesis of isolated chloroplasts and leaf disks at low concentrations in our last year's study, but the inhibition was an intermediate manner this year. The arsenical herbicides, cacodylate and DSMA, did not inhibit photosynthesis of isolated chloroplasts and leaf disks at 10^{-3} M last year, but they inhibited at 10^{-2} M slowly but definitely.

Amitrole was an enigma last year; in various types of screening methods it did not cause much inhibition. This year's work uncovered that this compound had an ability to act on the mechanism of stomatal opening, though slowly. As indicated last year, diquat acts more closely in the reactions involving photosystem I and/or CO_2 fixation; the lack of effectiveness in chloroplast photosynthesis may be caused by the assay techniques employed (see Zweig, 1965). Although the difference in the speed of action between last year's and this year's results is small, it emphasizes one point; namely, the relatively slow inhibition observed this year indicates that this compound is taken up by the root and subsequently translocated to the mesophyll chloroplasts slower than for example atrazine and bromacil. The relative ineffectiveness observed for the arsenical herbicides may likely be caused by the conversion from the biochemically inactive pentavalent form to the biochemically more active trivalent form in vivo (Webb, 1966). These differences are generally very minor, particularly when one compares the purposes of study of the two different years; last year's studies emphasized the immediate and direct action of herbicides at the site of action, while in this year's analysis herbicides were applied to the root and their effects were observed in a single leaflet of the plant.

These considerations, both general agreement and discussions of minor differences of the results obtained last year and this year, can lead to the following conclusions: (a) this year's study emphasizes how a given herbicide acts in vivo, including characterististics involved in uptake, translocation, and metabolism of the compounds in vivo, (b) the gas analyzer can provide quite accurate information as to whether a given compound inhibits mesophyll photosynthesis or not, and (c) the simultaneous use of a hygrometer allows to uncover a possible action of a herbicide on stomatal opening, as clearly indicated by the action of amitrole. In essense, simultaneous measurements of photosynthesis and transpiration employed this year are superior to diagnose the modes of action of photosynthetic inhibitors in intact plants.

EVALUATION OF METHOD

Once the instrumentation and assay procedures are established, the semiautomated instrumentation we developed detects quite accurately the modes of action of herbicides under relatively natural conditions. It further provides information as to where the site of phytotoxic action of a herbicide might be, on mesophyll photosynthesis or on the mechanism of stomatal opening.

Perhaps, one limitation in our procedure exists in the fact that the plant under study must be brought to a condition for our assay. Although the preconditioning regime has been established for young soybean plants, we have not tested if the same regime works for other plants.

Any instrumentation needs further improvements. The instrument used here has a response time of about 1 minute. The response time could be reduced by faster circulation of gas through the instrument and by reducing the total volume of the gas line. In order to run the instrument for a long time, the construction of the cold trap could be changed. Perhaps, the major improvement is in the further automation of data processing process: currently, the procedure has been established and a relatively simple operation is needed to obtain the data from the instrument, but the calculation of the data takes time. Computerization in data processing would certainly help the time consumption for analysis of the data.

ACKNOWLEDGEMENTS

For the construction of the instrument, Drs. John Tenhunen and Conrad S. Yocum gave us invaluable advice. We greatly appreciated their generous help.

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CHAPTER III

SIMULTANEOUS MEASUREMENTS OF RESPIRATION AND ION UPTAKE BY THE ROOT

By Walter R. Taylor and Hiroshi Ikuma

INTRODUCTION

The major physiological functions of plant roots can be thought of supplying necessary ions and water to the rest of the plant. Since water uptake by the root takes place by osmosis, the water potential of the root cells must be lowered for osmotic water uptake to occur. One way by which the water potential of the root cells is lowered is to lower the osmotic potential of the root cells by means of active transport of ions from the surrounding soil water into the cells. For active transport of ions, the cellular energy generated by respiration is utilized.

From the work performed during the preceding year with isolated corn root tips, it has become clear that (a) a few herbicides inhibited root respiration, and (b) several herbicides acted to inhibit the uptake of radioactive precursors of nucleic acids and protein. Since the precursors of nucleic acids and protein are rarely present in the soil water, several herbicides may act to inhibit ion uptake.

We have thus made attempts to measure the activities of respiration and ion uptake in the intact root system of a young soybean plant simultaneously. Root respiration was measured by both an oxygen electrode and a carbon dioxide electrode. Ion uptake activities were attempted to be analyzed by a potassium and/or nitrate electrode. As will be noted in this chapter, our effort to measure ion uptake by ion specific electrodes was not successful.

MATERIALS AND METHODS

Soybean plants used for this work were conditioned in an identical manner as those used in the work of CHAPTER II; the roots of 8-week old soybean plants were de-soiled and placed in aerated ½ strength Hoagland nutrient solution for about one week before use. The plant acclimatized in solution culture was brought into the laboratory, and the root was placed in a baby food jar (4½ oz. size) with the fresh ½strength Hoagland solution. The baby food jar was placed above a magnetic stirrer and the root solution was stirred as shown in Fig. III-1. The electrodes were inserted through the rubber stopper (size #9) which made the cuvette air-tight during measurements. The plant shoot was held upright through a small hole in the center of the rubber stopper (see also Fig. III-2).

For the measurements of root respiratory activity, an oxygen electrode and a carbon dioxide electrode were used in a manner similar to the work of the preceding year (see also Figs. III-1 and III-2).

For the root ion uptake study, potassium and nitrate electrodes were used (Fig. III-2), because potassium and nitrate are among the major ions needed for plant nutrition. The electrodes were first tested individually according to the recommendations by the manufacturer (Orion Research Inc., Cabridge, Massachusetts); both electrodes showed electrode potentials proportional to the logarithm of cencentrations of ions between 10^{-5} and 10^{-3} M when they were connected to Radiometer pH meters (PHM 71, Mk 2).. To record ion concentrations, pH meters were connected to Heath-Schlumberger recorders (SR-255B).

All electrode set-ups were operated in a floating mode; i.e., grounding of reference electrode terminals resulted in interference by other electrodes. During the period of measurements (20-30 minutes) the cuvette (baby food jar) was stirred but kept air tight, and in between measurements the root was aerated by bubbling air through for about 5 minutes to avoid anoxia.

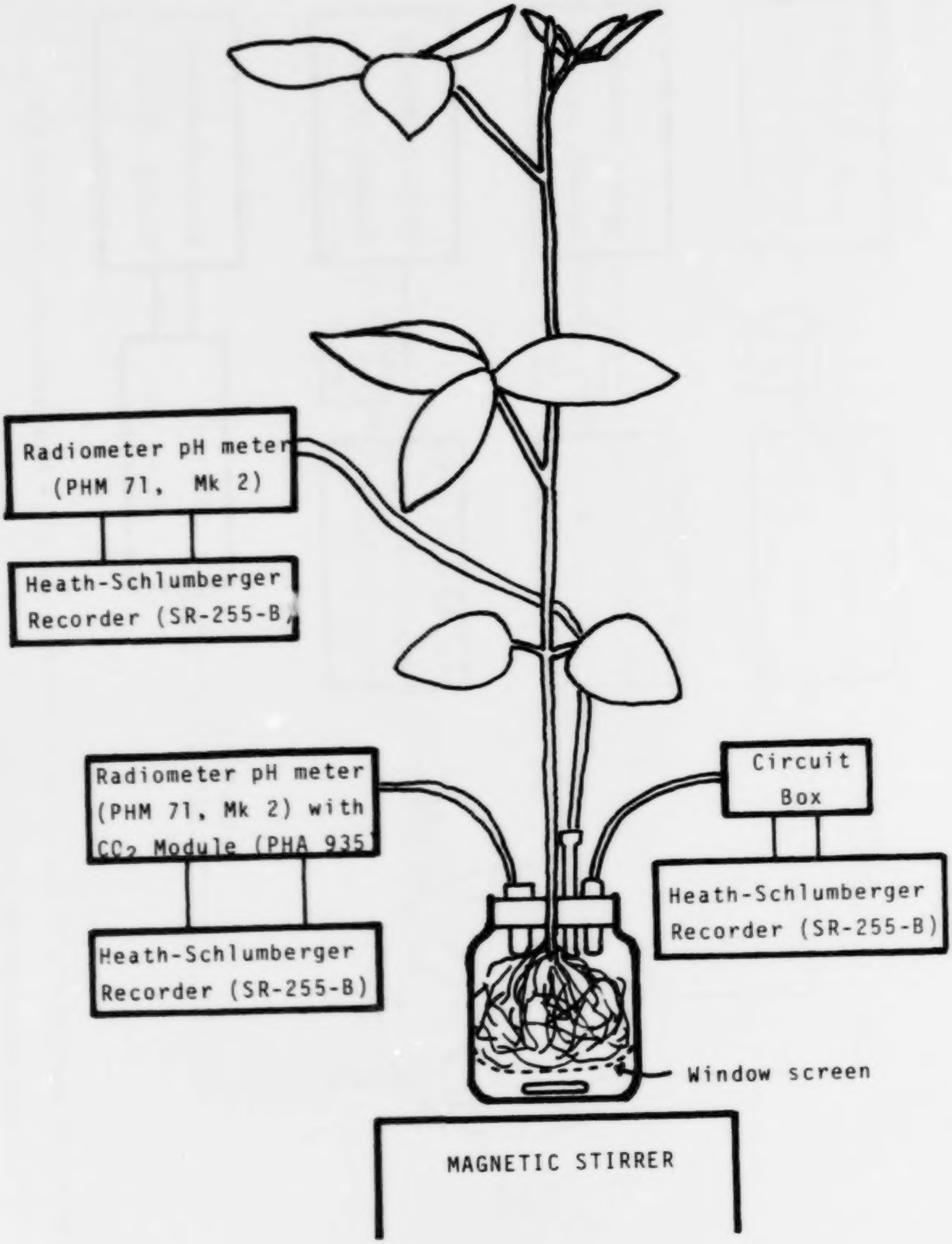

Radiometer pH meter
(PHM 71, Mk 2)
Heath-Schlumberger
Recorder (SR-255-B)
Radiometer pH meter
(PHM 71, Mk 2) with
CO2 Module (PHA 935)
Heath-Schlumberger
Recorder (SR-255-B)
Circuit
Box
Heath-Schlumberger
Recorder (SR-255-B)
Window screen
MAGNETIC STIRRER

Fig. III-1. The set-up used in the measurements of oxygen consumption, CO_2 evolution, and ion uptake activity by the intact soybean root.

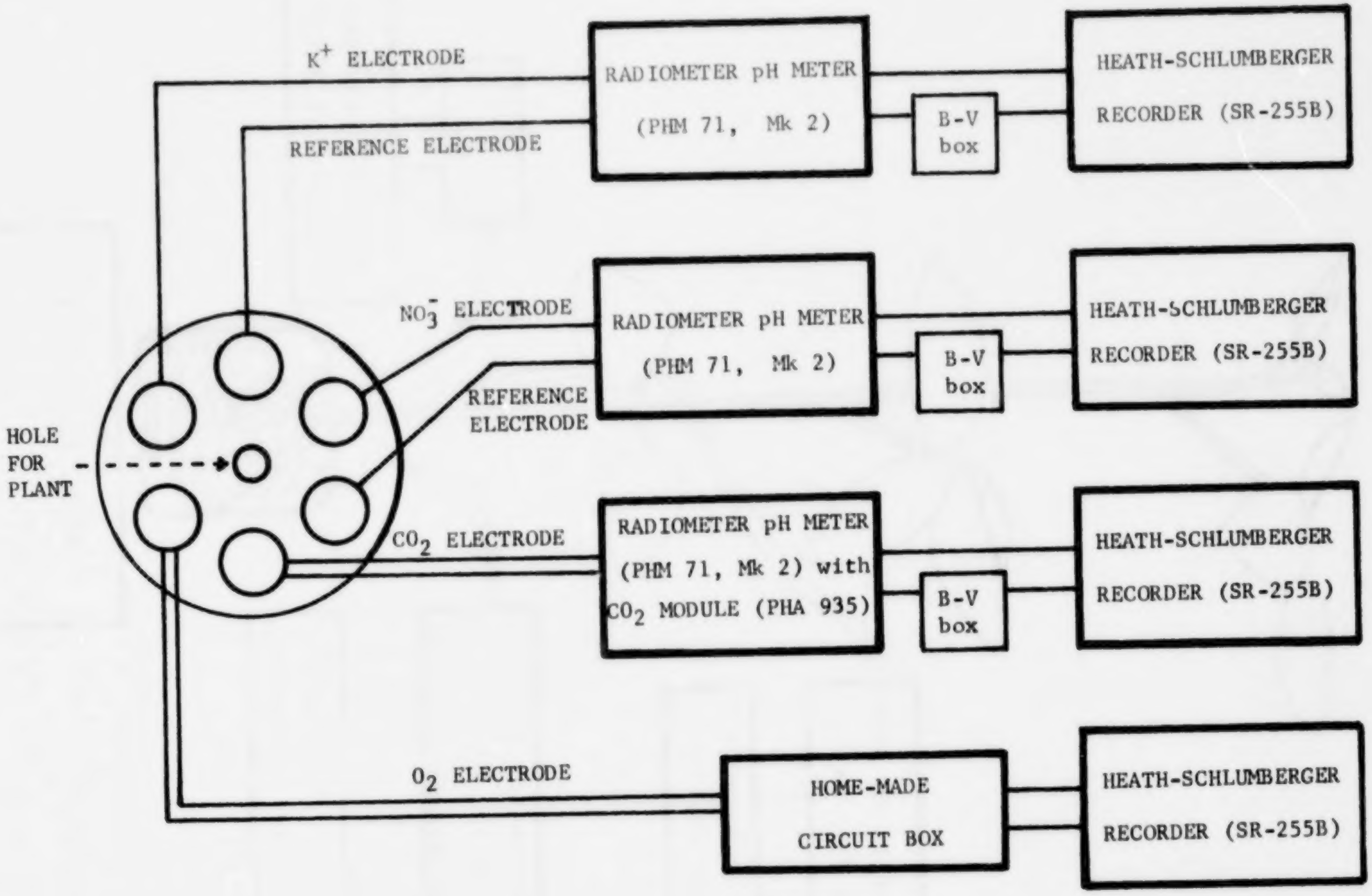

K$^+$ ELECTRODE
REFERENCE ELECTRODE
RADIOMETER pH METER
(PHM 71, Mk 2)
B-V box
HEATH-SCHLUMBERGER
RECORDER (SR-255B)
NO$_3^-$ ELECTRODE
REFERENCE ELECTRODE
RADIOMETER pH METER
(PHM 71, Mk 2)
B-V box
HEATH-SCHLUMBERGER
RECORDER (SR-255B)
HOLE FOR PLANT
CO$_2$ ELECTRODE
RADIOMETER pH METER
(PHM 71, Mk 2) with
CO$_2$ MODULE (PHA 935)
B-V box
HEATH-SCHLUMBERGER
RECORDER (SR-255B)
O$_2$ ELECTRODE
HOME-MADE
CIRCUIT BOX
HEATH-SCHLUMBERGER
RECORDER (SR-255B)

Fig. III-2. An overhead view of the cuvette (a baby-food jar) and a diagramatic presentation of the recording system with 6 electrodes.

RESULTS AND DISCUSSION

A. PERFORMANCE OF ION SPECIFIC ELECTRODES

When simultaneous measurements were made with the instrumentation diagramatically shown in Fig. III-2, ion uptake activity was not detected while active respiration was recorded by both oxygen and carbon dioxide electrodes. This unexpected situation posed a problem as to whether the plant root was not carrying out ion uptake actively or the electrodes were not measuring ion exchange sensitively. We have therefore performed many experiments if the problem could be rectified. In our varied, repeated attempts, the detection of ion uptake activity by these electrodes was not successful. In the paragraphs to follow, we summarize our attempts and effort briefly.

Either in the presence or in the absence of the root in the cuvette, both electrodes, used singly or in combination, responded in an expected manner to changes in KNO_3 concentrations. The electrodes themselves were functioning properly. When the ½ strength Hoagland solution was replaced by distilled water, the electrodes detected a very slow increase in both potassium and nitrate ions in the root solution, indicating a very slow leakage of these ions from the root. However, when the ½ strength Hoagland solution was replaced by a full strength or a 1/10 strength Hoagland solution, there was no change in ion concentrations. From these sets of experiments, it has become clear that the ion uptake activity by the root was much smaller than expected.

In order further to examine if the root took up ions so slowly, we attempted to starve the plant root with ions; i.e., after de-soiling the roots were placed in aerated distilled water for a period of 10-15 days prior to use. These roots did not again take up ions appreciably for a period of 4-6 hours. We also tried to use soybean plants of several growing stages; from shortly after germination through a mature plant with developing fruits. The results were again negative. Our inability to detect any appreciable ion uptake was further compounded by our frequent observations during this period that the roots of intact soybean plants generated new secondary roots more vigorously in distilled water than in Hoagland solution during the week of acclimatization. Perhaps, the roots of soybean plants we used were loaded with necessary ions for growth and maintenance of root activities.

The sensitivity level of both electrodes was about 10 μM, which was somewhat less than the sensitivity of the oxygen electrode and a carbon dioxide electrode (2-5 μM). We also attempted to improve the sensitivity of the electrode recording system. Unfortunately, however, the amplification of signal to detect ion concentration changes of about 1 μM or less resulted in a large, random drift in recording traces. This drift was apparently generated in the recording system rather than in the electrode themselves. In various attempts, we consistently failed to improve the sensitivity of the instrumentation for ion uptake activity.

In an early phase of this investigation, we employed a pH electrode in place of ion specific electrodes. The Hoagland nutrient solution has a pH of 5.5 - 6. This pH of the solution did not change during a period of 4-6 hours. If a small amount of an acid or alkali was added, the pH of the solution returned to near 6 in 10-20 minutes in the presence of the root. This tendency of the root to maintain the pH of the solution near 6 was not only found when the root was in the Hoagland solution but also when it was placed in distilled water. This buffering action of the root was unexpected and we think it is associated with our inability to detect ion uptake activity.

From our repeated failure to record consistent potassium and nitrate uptake, we concluded that (a) the electrode set-up was not sensitive enough to record ion uptake accurately during the 2-4 hour measuring period, and (b) soybean roots are not taking up ions vigorously. Although simultaneous measurements of ion uptake with respiration measurements of the roots are an attractive idea to assess the physiology of the root, we abandoned the use of ion specific electrodes because of the aforementioned repeated failure for a period of 6-8 months. In our examination of herbicidal effects on the root process, therefore, we used an oxygen electrode and a carbon dioxide electrode simultaneously for measurements of respiratory activity.

B. EFFECTS OF HERBICIDES ON ROOT RESPIRATION

The simultaneous measurement of oxygen and carbon dioxide concentrations in the root solution was made in the manner illustrated in Figs. III-1 and III-2. The recording traces obtained were practically identical to those obtained with isolated corn root tips (see Fig. V-1 of the Final Report of the preceding year). A small amount of herbicide stock solution (prepared in DMSO for water insoluble herbicides and in distilled water for water soluble herbicides) was added to the root solution to give a concentration of 10^{-3}, 10^{-4}, or 10^{-5} M, after steady rates of respiration were obtained with the electrode. The concentration of DMSO carried into the root solution was 10% or less. DMSO alone (1% or 10%) appeared to increase the respiratory rates temporarily for about 30 minutes (see Fig. II-3), but the rates fell later to the original value. At about 2 hours after DMSO addition, the respiratory rates were identical to those immediately before its addition. The effects of 15 herbicides on both oxygen consumption and CO_2 evolution are summarized in Table III-1, where the effects are presented in terms of % of control values in the last two columns.

Table III-1

Effect of 15 herbicides on the respiratory activity of the roots of intact soybean plants

The respiratory activity was measured with an oxygen electrode and a CO_2 electrode for a 1-2 hour period, then a herbicide solution was added, and respiratory rates were measured for about 2 hours. From the control rates RQ values were calculated, and herbicide effects are expressed in terms of % of control rates. Plants used were grown for 6-8 weeks in the greenhouse and had 1 or 2 fully grown trifoliolate leaves. The root was desoiled, acclimatized in the liquid culture conditions, and bathed in 150 ml of 1/4 strength Hoagland nutrient solution.

Herbicide	Conc. used	Before Herbicide addition			After Herbicide addition	
		O_2 Uptake	CO_2 Evolution	RQ	O_2 Uptake	CO_2 Evolution
	(M)	(nmoles/min·plant)		(CO_2/O_2)	(% of control)	
Amitrole	10^{-3}	410	503	1.23	118	108
Atrazine	10^{-3}	584	714	1.22	115	104
Bromacil	10^{-3}	464	480	1.03	110	108
Cacodylate	10^{-3}	291	330	1.13	0	0
	10^{-4}	270	266	0.99	73	88
Chlorpropham	10^{-3}	202	228	1.13	157	156
	10^{-4}	414	391	0.94	104	83
2,4-D	10^{-3}	473	643	1.36	46	57
	10^{-4}	439	500	1.14	98	105
Diallate	10^{-3}	276	346	1.25	89	98
Diquat	10^{-3}	652	674	1.03	92	115
Diuron	10^{-3}	579	679	1.17	124	105
DNOC	10^{-3}	383	359	0.94	0	0
	10^{-4}	435	458	1.05	283	222
	10^{-5}	375	305	0.81	147	126
Karbutylate	10^{-3}	419	344	0.82	177	136
	10^{-4}	388	363	0.94	103	98
Monuron	10^{-3}	409	517	1.26	95	80
Propham	10^{-3}	253	200	0.79	104	134
Simazine	10^{-3}	314	392	1.25	121	114
Trifluralin	10^{-4}	628	722	1.15	112	96

As noted in the O_2 uptake and CO_2 evolution columns before herbicide addition, observed rates of respiration by intact soybean roots are different for individual plants. Even after calculations on the basis of root fresh weight and dry weight, large differences were noted. Because of this, we have to record the rates before and after herbicide addition and to use the rates before herbicide as the control values. All the rates expressed in this Table are averages of two or more readings. Average experimental errors are about 30%, similar to our experiments of last year with isolated corn root tips. We thus consider the rates after herbicide addition beyond 30% to be of significance. The RQ value for soybean roots is expectedly about 1.0; an average value of all RQ determinations in this table is 1.05. As seen in RQ values, these determinations also carry about 30% experimental error. The major source of these errors is quitely likely in the determination of CO_2 evolution rates, because the CO_2 electrode has a much longer response time than the O_2 electrode and is not as stable as the O_2 electrode (see Fig. V-1 of the Final Report of the preceding year).

The addition of a herbicide caused either no effect, inhibition, or stimulation of respiratory rates. Of the 15 herbicides tested, 10 herbicides caused no noticeable effects at the highest concentrations used; they are amitrole, atrazine, bromacil, diallate, diquat, diuron, monuron, simazine, and trifluralin.

At 10^{-3} M, cacodylate and DNOC caused strong inhibition of both O_2 uptake and CO_2 evolution. The inhibition by cacodylate disappears at 10^{-4} M, but DNOC stimulates respiration 2 to 3 fold at 10^{-4} M. Some stimulation can be seen even at 10^{-5} M DNOC. In view of our earlier work with isolated mitochondria and isolated corn root tips, DNOC stimulation at 10^{-4} and 10^{-5} M must be due to uncoupling of oxidative phosphorylation (see Figs. II-3 and

II-4 and Table V-1 of the Final Report of the preceding year). At 10^{-3} M, this herbicide inhibits mitochondrial electron transport. Cacodylate did not cause any effect at 10^{-3} and 10^{-2} M on the respiratory activities of isolated mitochondria or those of isolated corn root tips (see the Final Report of the preceding year). The inhibition of respiration observed here with intact soybean roots may indicate that soybean roots convert metabolically inert pentavalent arsenical in cacodylate into a metabolically inhibitory trivalent form.

Chlorpropham and karbutylate stimulate respiration moderately at 10^{-3} M, but not at 10^{-4} M. Both herbicides caused no detectable stimulation of respiration in isolated mitochondria nor isolated corn root tips (see the Final Report of the preceding year). Perhaps, the observed stimulation is due to metabolic products of these herbicides in the roots which act as weak uncouplers. 2,4-D caused moderate inhibition at 10^{-3} M, but not at lower concentrations. Since this herbicide causes massive cell damage in the root at 10^{-3} M (see the Final Report of the preceding year; also CHAPTER VI), the respiratory inhibition must be due to general disintegration of the root cells at this high concentration.

It should be noted that all herbicides tested caused the same effects for both O_2 uptake and CO_2 evolution. Although the idea of using two electrodes came from possible differential effects of herbicides on electron transport activity (as detected by the O_2 electrode) and on other metabolic activities evolving CO_2 (as detected by the CO_2 electrode), the agreement in results from both electrodes suggests that the use of one stable electrode (i.e., the O_2 electrode) is enough to detect herbicidal action on root respiration.

EVALUATION OF METHOD

Since ion uptake by the root cells is presumably an active process utilizing the cellular energy derived from respiratory activities, simultaneous analyses of both respiration and ion uptake were of interest to assess the physiological state of the root. We thus tried to monitor such activities with 4 electrodes simultaneously.

Our repeated attempts to detect ion uptake activity with two ion specific electrodes failed. Our results strongly suggested that bilogical problems primarily caused the failure, while instrumentation problems were not altogether eliminated. This situation caused us to abandon the use of ion specific electrodes for our analysis of herbicidal effects on root processes.

Respiratory activities of the root were analyzed with the same O_2 and CO_2 electrodes as those used in the work of the preceding year, and the herbicidal effects detected by the two electrodes were practically identical to those obtained during the preceding year. Fro the two years' study, two points emerge: (a) both electrodes give rise to the same results, and (b) an O_2 electrode is a more stable instrument than an CO_2 electrode.

Analyses of instrumental and biological problems associated with the analysis of the root processes lead us to a conclusion that the measurement of root respiration by means of a stable O_2 electrode alone would provide us with the results to assess the physiological state of the root. In addition, many herbicides tested are photosynthetic inhibitors and only a few, most notably DNOC, caused significant inhibition of root respiration. In the analysis of the overall plant processes, an emphasis is better placed on the analysis of the shoot processes, particularly the processes associated with mesophyll photosynthesis.

In our analysis of root respiration, we employed a manual procedure of aeration in between measurements. This aeration procedure may be made automatically, if the oxygen addition to the root solution is made by electrolytically and the electrolytic supply of oxygen and respiratory measurements can be automated. Such a scheme was considered, but not practiced. In addition, automated analysis of respiratory rate would certainly help the efficiency of operation.

CHAPTER IV

DEVELOPMENT OF SIMULTANEOUS MEASUREMENTS OF ROOT AND SHOOT PROCESSES

By Howard H. Robinson, Walter R. Taylor, and Hiroshi Ikuma

INTRODUCTION

The major purpose of this entire project is to arrive at a single method of analysis of the mode of action of herbicides in intact plants. Such a method must combine several techniques, since any individual technique would not be sufficient to pinpoint herbicidal modes of action. From the work performed in the preceding year, it has become clear that analyses with isolated organelles and isolated tissues are not sufficient to pinpoint the primary mode of action of any herbicides, although the mode of action on individual metabolic processes would be analyzed in depth. For the work of the present year, we have thus far endeavored to analyze and develop relatively simple techniques to examine primary processes in the shoot and in the root separately. Our results have been encouraging, particularly in the analysis of the shoot processes; our results not only confirm the data obtained with isolated chloroplasts and with leaf disks of the preceding year, but also they extend the analysis into the herbicidal action on stomatal opening. Our analysis of the root process provided the data which generally confirm the results obtained with isolated corn root tips and with isolated mung bean mitochondria, while our analysis of ion uptake by the root was a failure.

From our analyses of the two successive years, we felt a strong possibility of combining the analytical techniques for the shoot and the root processes. In this chapter, we report the performance of such a combined, simultaneously measuring technique.

COMBINED INSTRUMENTATION

Figure IV-1 shows the combined method schematically and Fig. IV-2 illustrate the instrumentation in a photograph. In both figures, the labels indicate corresponding components. Since the analysis of root respiration with both an O_2 electrode and a CO_2 electrode gave essentially the same results, only the oxygen electrode was employed in this combined analysis for the analysis of root respiration. An oxygen electrode was inserted in the root solution (N) and the oxygen concentration was recorded on a Heath recorder (R-s). The middle leaflet of the second trifoliolate leaf was sealed in the leaf chamber (A). The output from this chamber was connected to a hygrometer (F), to a solenoid valve (G), to a cold trap (I), to a heat exchanger (K), to a gas analyzer (L), and back to the leaf chamber via a peristaltic pump (M). Recorders were connected to the hygrometer (R-2) and to the gas analyzer (R-1). The leaf chamber was illuminated with a high intensity Oriel lamp (C) through a heat filter (B). The rest of the plant was illuminated with a 150 watt photoflood lamp (E) and aerated by means of a fan. As in our shoot analysis, the solenoid switch valve (G) allowed the whole gas circuit to be closed or exposed to the external air. The peristaltic pump allowed the gas circulation to proceed closkwise in Fig. IV-1. The solenoid valve was electrically controlled to open or close automatically at a 3 minutes' interval.

RESULTS AND DISCUSSION

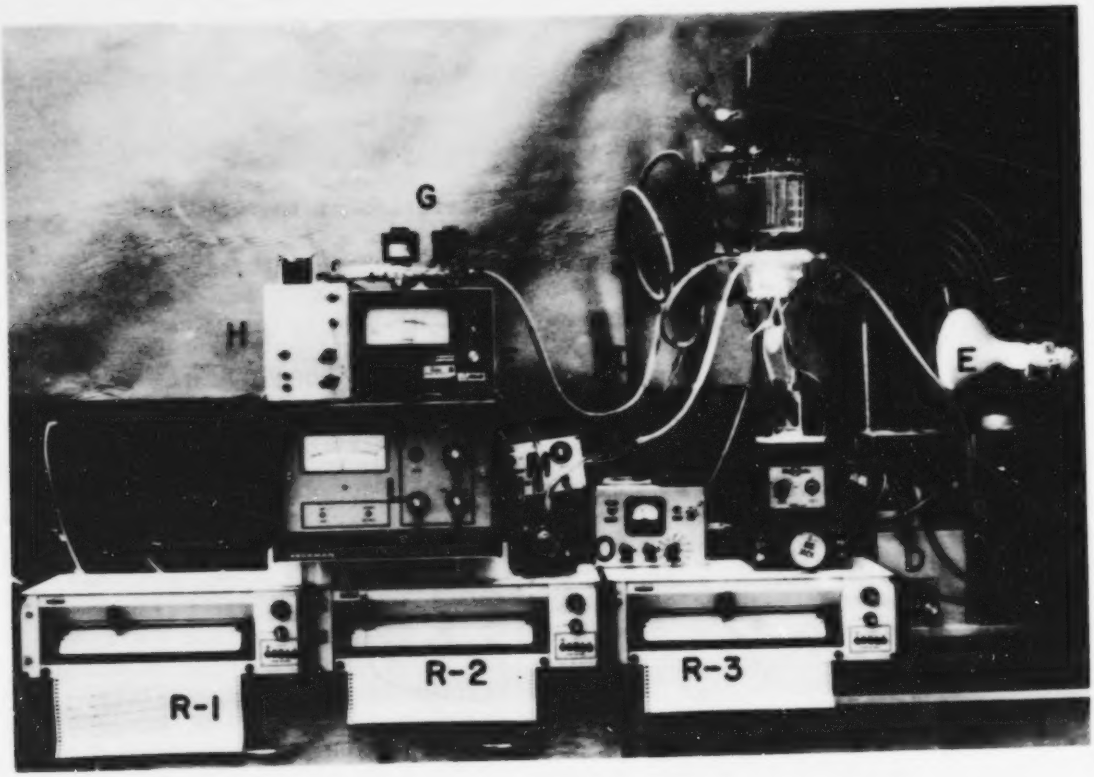
The combined analytical method was applied to three types of herbicides; (a) a herbicide which primarily inhibits photosynthesis, (b) a herbicide which inhibits root respiration, and (c) a herbicide which presumably does not act on both root respiration and leaf photosynthesis.

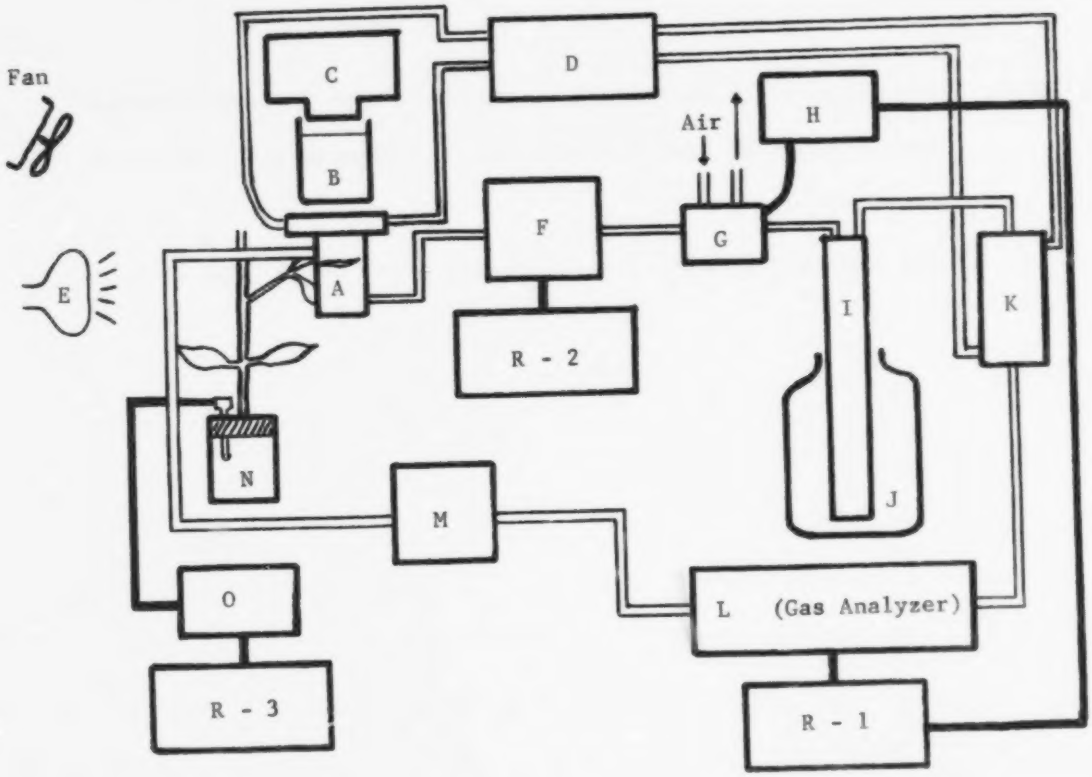
Fig. IV-1. (Bottom figure) Schematic presentation of the combined instrumentation for analyses of leaf photosynthesis, transpiration, and root respiration.

A, leaf sample chamber. B, heat filter (600 ml beaker with distilled water). C, Oriel tungusten lamp. D, constant temperature circulator (Lauda Thermostat, Type K2-R). E, 150 watt photoflood lamp. F, thermoelectric dew point hygrometer (Cambridge Systems Inc., Model 880). G, solenoid valve. H, electrical control box (see Fig. II-2). I, cold trap. J, dry-ice ethanol container to keep the cold trap temperature at -56° C. K, heat exchanger. L, infrared CO_2 gas analyzer (Beckman gas analyzer, Model 864). M, peristaltic pump (Cole-Parmer Masterflex tubing pump). N, lucite container to house the root system of the plant and an oxygen electrode (although not shown in this diagram, a stirrer is placed immediately below this container to stir the root solution). O, circuit box for the oxygen electrode. R-1, R-2, R-3; Heath-Schlumberger recorders to record readings of the gas analyzer, hygrometer, and oxygen electrode, respectively.

Fig. IV-2. (Top figure) A photograph of entire set-up for simultaneous measurements of leaf photosynthesis, transpiration, and root respiration.

The same alphabetical notations are given as in Fig. IV-1.


G
H
E
R-1
R-2
R-3


Fan
C
D
B
H
Air
G
F
A
E
I
K
R - 2
N
M
J
O
L (Gas Analyzer)
R - 3
R - 1

For the analysis of the action of herbicides which primarily inhibit photosynthesis, we chose 10^{-3} M atrazine. Figure IV-3 illustrates the effect of 10^{-3} M atrazine on root respiration, mesophyll photosynthesis, and leaf transpiration. The upper figure illustrates the tracings from the three recorders, and the lower figure shows the calculated rates of respiration and photosynthesis and the same water vapor trace as the upper figure. Both the upper and the lower figures are drawn on the same time scale. Atrazine was added at the arrow for both figures.

It is clear from the figures that this herbicide inhibits photosynthesis (CO_2 uptake) by the mesophyll cells first, then stomatal opening (H_2O trace), while it has no effect on root respiration during the 80 minute's period after the herbicide addition. A slight increase in photosynthesis and respiration shortly after the addition of atrazine can be ascribed to the addition of 10% DMSO which accompany atrazine (cf. Figs. II-3 and II-5).

It can be concluded that this herbicide acts primarily on mesophyll photosynthesis. Its inhibition of mesophyll photosynthesis brings about inhibition of stomatal opening subsequently (see CHAPTER II). It is of interest that the inhibition of photosynthesis is so strong that the leaf cells show respiration after 40 minutes from the addition of this herbicide. The lack of its effect on root respiration has been documented (see the Final Report of the preceding year and Table III-1 of this report).

For illustration of the mode of action of a herbicide which primarily inhibits root respiration, we employed 10^{-3} M DNOC, as shown in Fig. IV-4. Again the upper figure shows the actual recorder traces and the lower figure calculated rates with the time axis (abscissa) the same for both figures. This herbicide at this high concentration inhibits the mitochondrial electron transport (see Fig. II-3 of the Final Report of the preceding year and Table III-1 of this year's report), then mesophyll photosynthesis (see Tables III-1

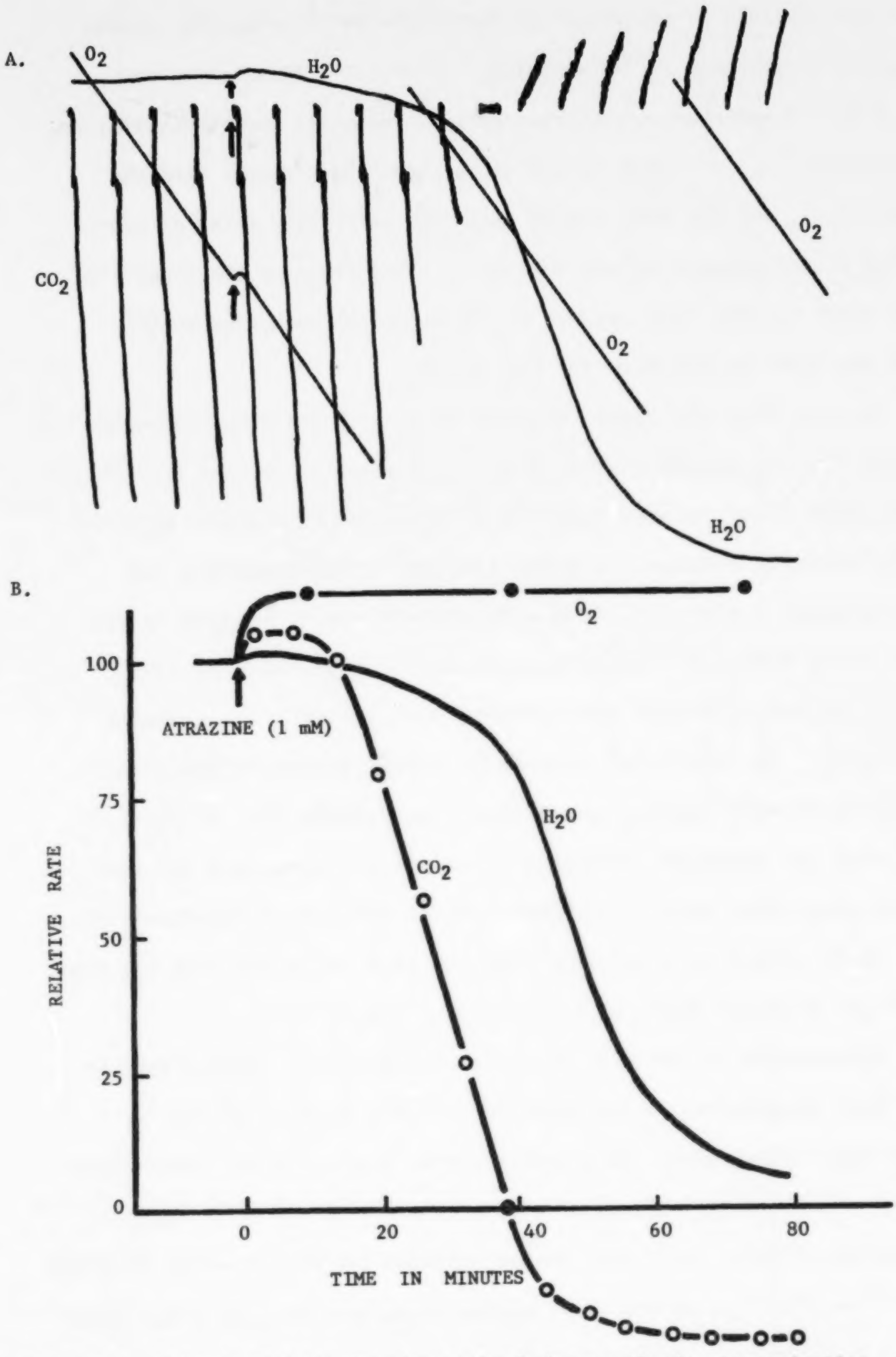


Fig. IV-3. Effects of 1 mM atrazine on leaf photosynthesis, transpiration, and root respiration as detected by the combined instrument.

A, a copy of tracings from three recorders. B, calculated rates of photosynthesis and respiration are plotted with the transpiration results.

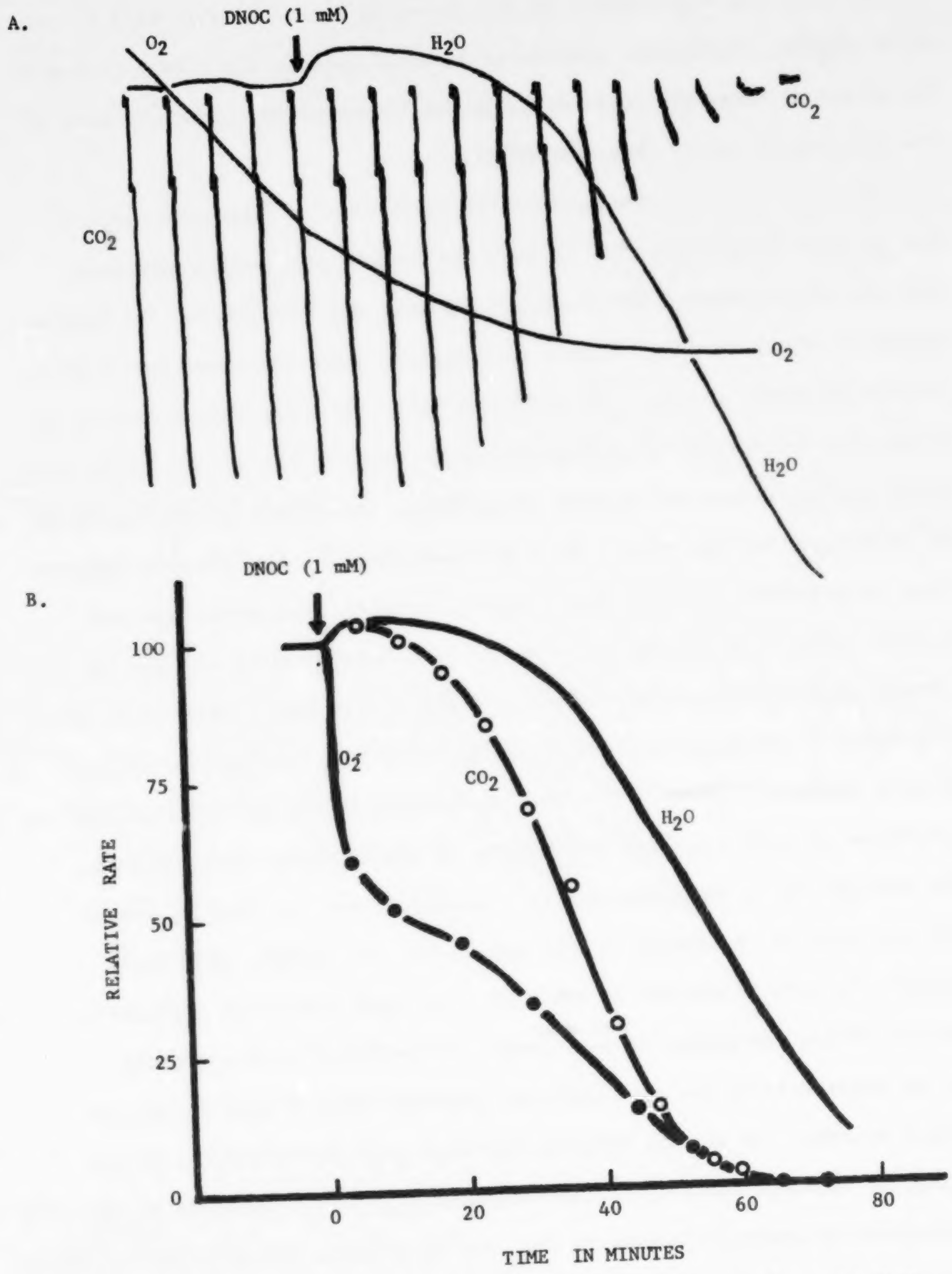


Fig. IV-4. Effect of 1 mM DNOC on leaf photosynthesis, transpiration, and root respiration as detected by the combined instrument.

A, a copy of tracings from three recorders. B, calculated rates of photosynthesis and respiration are plotted with the transpiration results.

and III-2 of the Final Report of the preceding year and Table II-3 of this years report). Inhibitory effects of stomatal opening are likely caused by its effect on mesophyll photosynthesis and increased CO_2 concentrations in the substomatal cavity (see CHAPTER II).

Amitrole did not cause appreciable inhibition in chloroplast photosynsis, in root respiration, and in root tip nucleic and protein synthesis (see the Fignal Reports for the preceding year and this year). It, however, showed an effect to close stomata more or less about the same time it would inhibit mesophyll photosynthesis (CHAPTER II). We chose this herbicide to illustrate the effects of a herbicide whose primary action was not on mesophyll photosynthesis nor on root respiration. The effect of this herbicide is illustrated on Fig. IV-5. This herbicide does not inhibit root respiration, as expected. It inhibits, however, mesophyll photosynthesis and stomatal opening in a manner similar to the effect of atrazine, but its effects appeared very slowly indeed. Although this figure shows that the inhibition of photosynthesis comes earlier than that of stomatal opening, we have repeatedly observed in other studies (as in CHAPTER II) that the inhibition of both processes takes place at approximately the same time. The discrepancy in the appearance of the effects may also be ascribed to the fact that the treatment of the root system was somewhat different between the work in CHAPTER II and here: the root system was vigorously aerated during the period of measurement in CHAPTER II studies, while in the present study the root aeration appeared every 30 minutes and for only 5 minutes. It should, however, be noted that the half-times of the mesophyll photosynthesis inhibition appeared much faster relative to the inhibition of stomatal opening in the case of atrazine and DNOC than in the case of amitrole. This comparison further supports the idea that the amitrole effect is different from that of atrazine or DNOC.

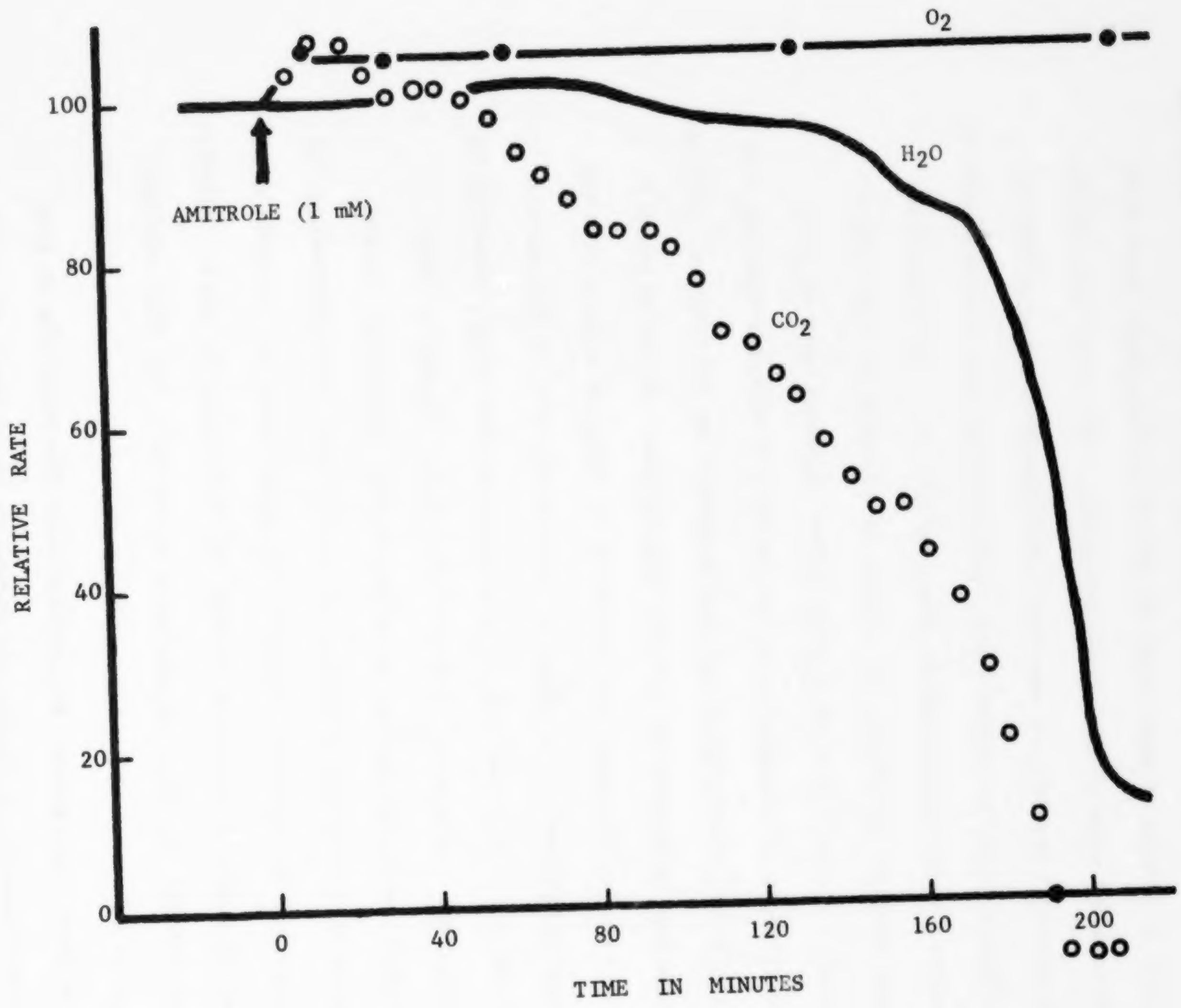


Fig. IV-5. Effect of 1 mM amitrole on leaf photosynthesis, transpiration, and root respiration.

The combined analytical technique described here detects the herbicidal action on root respiration, mesophyll photosynthesis, and leaf transpiration rapidly and simultaneously. An advantage with this method is the detection of the order of action of herbicides on the three processes, from which one can deduce the mode of action of herbicides. If a herbicide exerts its phytotoxicity primarily on mesophyll photosynthesis, its action on stomatal opening tends to appear later, particularly when the herbicide is administered at high concentrations (see CHAPTER II). If a herbicide acts on root respiration first, its action is detected in root oxygen uptake activity first. If a herbicidal effect appeares more or less simultaneously both on mesophyll photosynthesis and stomatal opening, one can deduce that its action would be more primarily on the stomatal opening.

The combined technique has certain limitations. It cannot detect the action of herbicides which act primarily on other processes than the three processes examined. For example, trifluralin acts at the mitotic processes, and its effect was not clearly detected when singly examined by the techniques for the shoot or the root processes. Secondly, this instrumentation cannot be applied to slowly acting herbicides, if the effects appear more than say 8 hours. In such a case, the construction of the cold trap must be improved. Even if the construction of the cold trap is improved, it would be difficult to run the instrument for days. Thirdly, it should be pointed out that the analysis of the data from this combined instrumentation take a sizable about of time.

Despite these limitations, we consider that the principle of this combined, simultaneously analyzing techniques to be of high value in assessing the phytotoxicity of herbicides. It readily detects the action of many herbicides which interferes with mesophyll photosynthesis, root respiration, and stomatal opening. It would serve as an initial screening device for many herbicides even for those whose action is on other plant metabolic processes.

EVALUATION OF METHOD

The combined method used in these investigations can not only detect the mode of those herbicides whose action is primarily on the inhibition of mesophyll photosynthesis, root respiration, and stomatal opening, but also provide the first screening device for other herbicides whose action does not belong to the three named processes. If a newly synthesized compound to be truly herbicidal, such a compound would inhibit truly plant processes; since the differences between plant and animal in many basic metabolic processes, other than photosynthesis and related pathways, are similar between animal and plant, a new compound would expectedly interfere photosynthetic processes. The proposed and tested technique is expected to detect such a new compound.

Not only from the tests performed, but from the expected development, we strongly feel that the combined technique or modification thereof would serve as the primary or initial screening technique.

The employed technique is obviously at its infancy in technology. It can and should be improved to save data processing steps as well as unnecessarily time-consuming manual steps. If we perfect such steps and correct inperfections which exist in the present set-up, the principle of the combined technique would certainly be incorporated in the screening work in the future.

CHAPTER V

FURTHER ANALYSIS OF PHYTOTOXICITY BY MEANS OF CHLOROPHYLL FLUORESCENCE: EFFECTS OF DIALLATE AND TRIFLURALIN

By Howard H. Robinson, Charles F. Yocum, and Hiroshi Ikuma

INTRODUCTION

Because chlorophyll fluorescence was expected to serve as a sensitive indicator of photosynthetic activity, we initiated the analysis of herbicide phytotoxicity by means of chlorophyll fluorescence in the preceding year using soybean leaf disks into which a herbicide was infiltrated (see CHAPTER IV of the Final Report of the preceding year). We thus constructed a leaf disk fluorometer (Fig. IV-1 in the Final Report of the preceding year) and examined the effects of 9 herbicides with our home-made instrument (Table IV-1 of the Final Report of the preceding year). There were several problems associated with the way by which fluorescence was examined, but the results generally agreed with what had been expected from the oxygen electrode assays on isolated chloroplasts.

In further exploration of the applicability of the chlorophyll fluorescence technique to the leaf of intact soybean plant, we continued this study during the present year. When we examined the effects of diallate and trifluralin with the same instrument of the preceding year, we obtained quite unexpected results. This situation prompted us to examine in detail the effects of these two herbicides on chlorophyll fluorescence with isolated chloroplasts.

MATERIALS AND METHODS

For the analysis of soybean leaf disk fluorescence, the same instrument and procedure as presented in CHAPTER IV of the preceding year were employed.

For studies with isolated chloroplasts, the fluorometer was modified in particular in the arrangement of the cuvette housing. The cuvette used was a regular 4 ml spectrophotometer cuvette with 4 clear sides, and it was placed in a jacket of water bath made of pieces of clear window glass. The jacket was connected to a temperature regulator (Haake Model FJ) and maintained at a temperature of 30^{o} C. The incident blue beam was shone on the cuvette and the fluorescent light was detected with a photomultiplier at 90^{o} with respect to the incident beam after passing through a secondary filter (Corning CS2-64). The recording of fluorescence was carried out with the same way as that for leaf disk fluorescence.

When oxygen evolution was measured simultaneously with the measurement of fluorescence, an oxygen electrode was inserted in the cuvette from above and the tip of the electrode was positioned immediately above the incident beam. The content of the cuvette was stirred by means of a stirrer both in the presence and in the absence of the oxygen electrode in the cuvette.

Chloroplasts were isolated in the same manner as reported in CHAPTER III of the Final Report of the preceding year.

RESULTS AND DISCUSSION

A. LEAF DISK FLUORESCENCE

The herbicides, diallate and trifluralin, showed unique effects on electron transport in isolated chloroplast (see CHAPTER III of the Final

Report of the preceding year): trifluralin inhibited electron transport between photosystems I and II as did diallate at apparently different sites. Diallate appeared to possess *a secondary activity* as a weak inhibitor of photosystem II and also to serve as an energy transfer inhibitor. It was thus expected that either herbicide would increase chlorophyll fluorescence as diuron would.

In our initial series of experiments, it was observed that diallate at 0.2 mM caused about 3-4 times higher fluorescence than diuron at the same concentration, but no increase in fluorescence was induced by addition of trifluralin. In repeated attempts, the degree of enhancement in fluorescence induced by diallate varied from experiment to experiment, while no fluorescence increase by trifluralin was repeatedly confirmed.

Figure V-1 illustrates a titration of diallate effects on leaf disk fluorescence as compared with the effect of diuron. Within the concentration range used, diallate is clearly less effective in causing chlorophyll fluorescence of leaf disks than diuron. In addition, this figure again indicates possible enhancement of fluorescence by diallate at higher concentrations over that by diuron.

In order to analyze further these unexpected results, we further examined the effects of these herbicides on chlorophyll fluorescence using isolated chloroplasts from spinach leaves.

B. CHLOROPHYLL FLUORESCENCE IN ISOLATED SPINACH CHLOROPLASTS

The Effect of Diuron. Similar to the work in the preceding year, diuron effect was considered as the control for the effects of diallate and trifluralin. Figure V-2 illustrates the effect of diuron. Addition of a chloroplast suspension to the illuminated cuvette causes a sharp rise

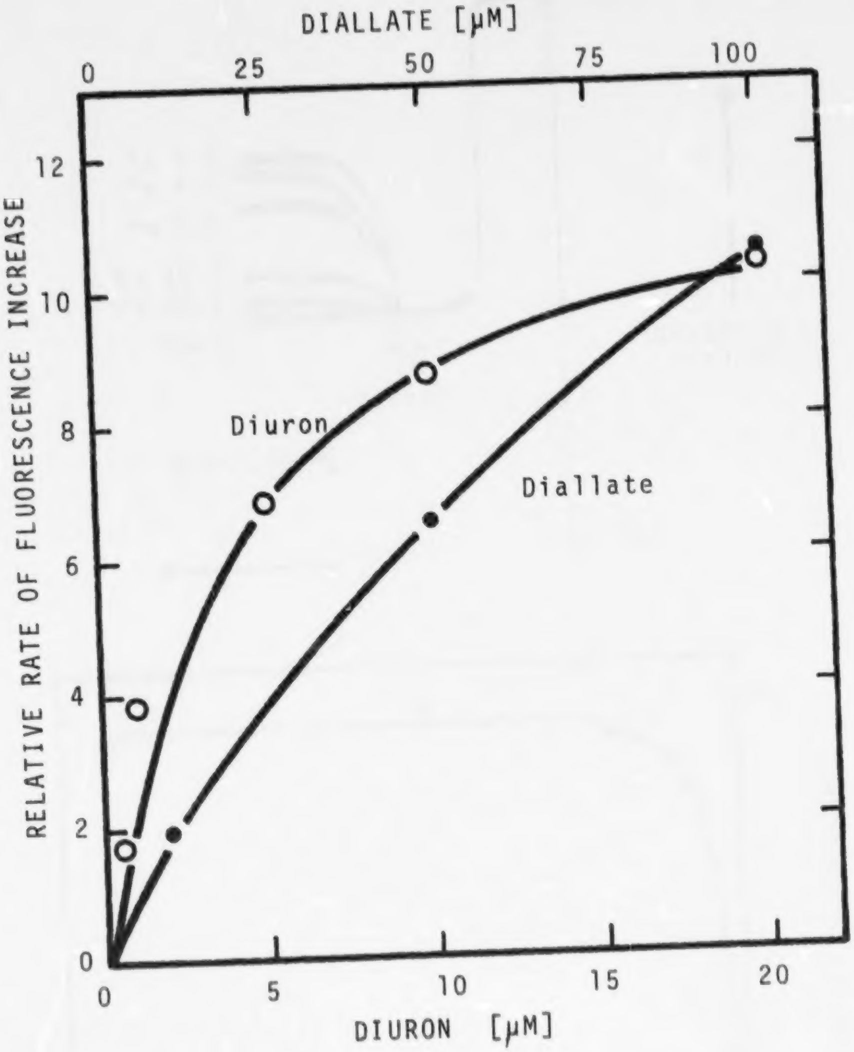


Fig. V-1. Effect of diallate and diuron on relative rates of fluorescence increase in soybean leaf disks.

Diuron curve is the same as that reported in the preceding year (Fig. IV-2 of the Final Report of the preceding year).

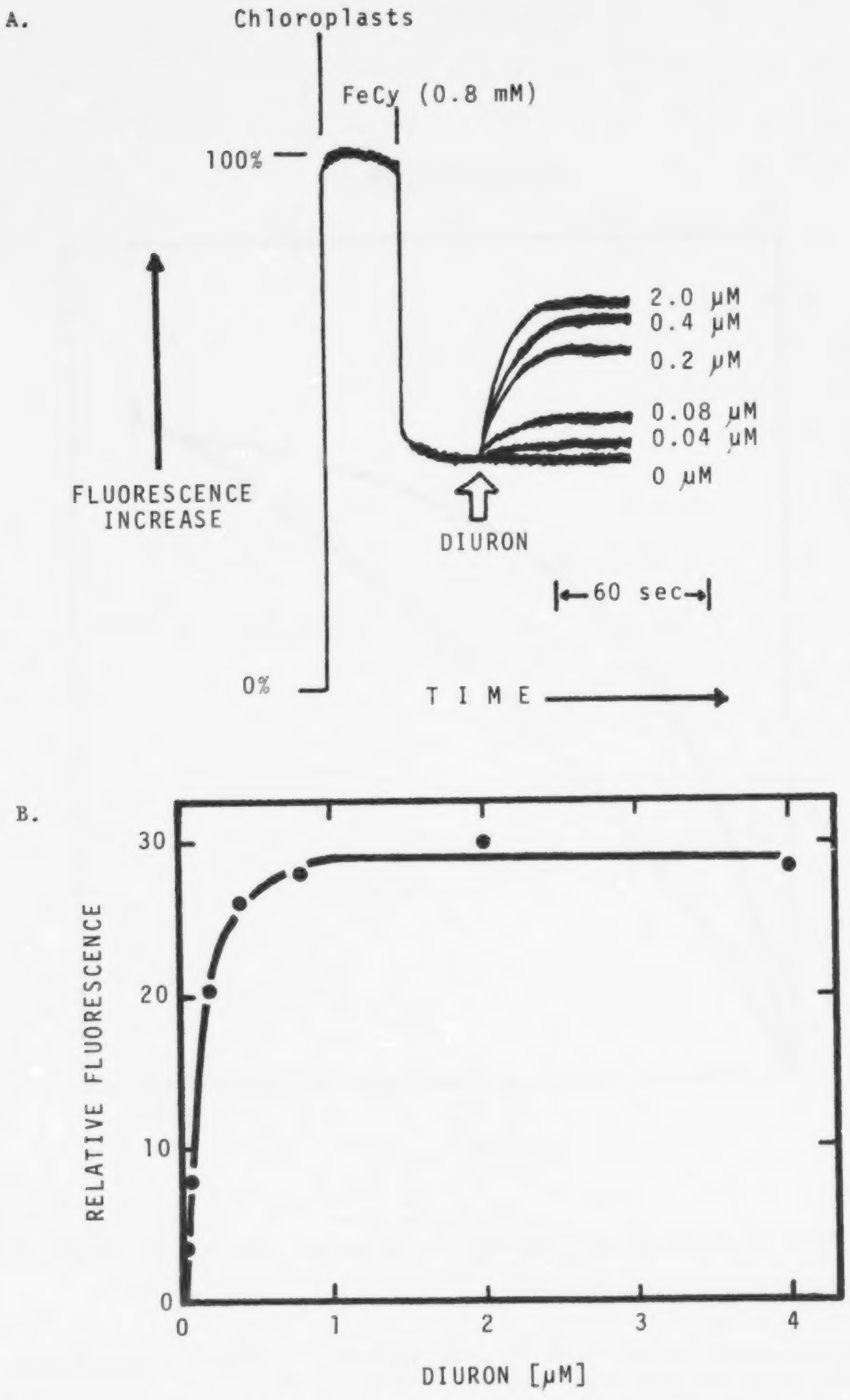


Fig. V- 2. Effect of diuron concentrations on fluorescence of isolated spinach Chloroplasts.

A, tracings from recorder. B, maximal fluorescence attained at respective diuron concentrations.

in fluorescence, subsequent ferricyanide addition decreases fluorescence by about 60%, and a subsequent addition of diuron increases fluorescence. The decrease in fluorescence after ferricyanide addition is expected because this compound initiates electron transport activity in chloroplasts. Since diuron inhibits the electron transport at photosystem II, it causes an increase in fluorescence. The extent of fluorescence increase after diuron addition is dependent upon its concentration (Fig. V-2A). If one plots the maximum fluorescence attained after diuron addition as the function of diuron concentrations (Fig. V-2B), a half-maximal effect is found at 0.15 μM. This titer value corresponds very closely with I_{50} value of diuron for oxygen evolution (see Fig. III-2A and Tables III-1 and III-2 in the Final Report of the preceding year). With diuron, therefore, the degree of chlorophyll fluorescence closely approximates the diuron effect on photosynthetic electron transport.

Effects of Diallate. If diallate was added after ferricyanide, the results (Fig. V-3A) resemble those of diuron. Closer examination reveals, however, that the kinetics of the fluorescence rise with diallate are different from those with diuron in two aspects: (i) at a low diallate concentration (20 μM) an initial rise of fluorescence is followed by a subsequent decrease, and (ii) at high concentrations the initial rapid rise is followed by a slow rise. It appears that diallate inhibition of photosynthetic electron transport is removed in time when a low concentration of diallate is given, while secondary inhibition appears to set in slowly at high concentrations.

If the maximum fluorescence attained by diallate treatment is plotted as a function of its concentration, the results (Fig. V-3B) indicates that a half-maximal effect is found at about 120 μM. It should be noted that the

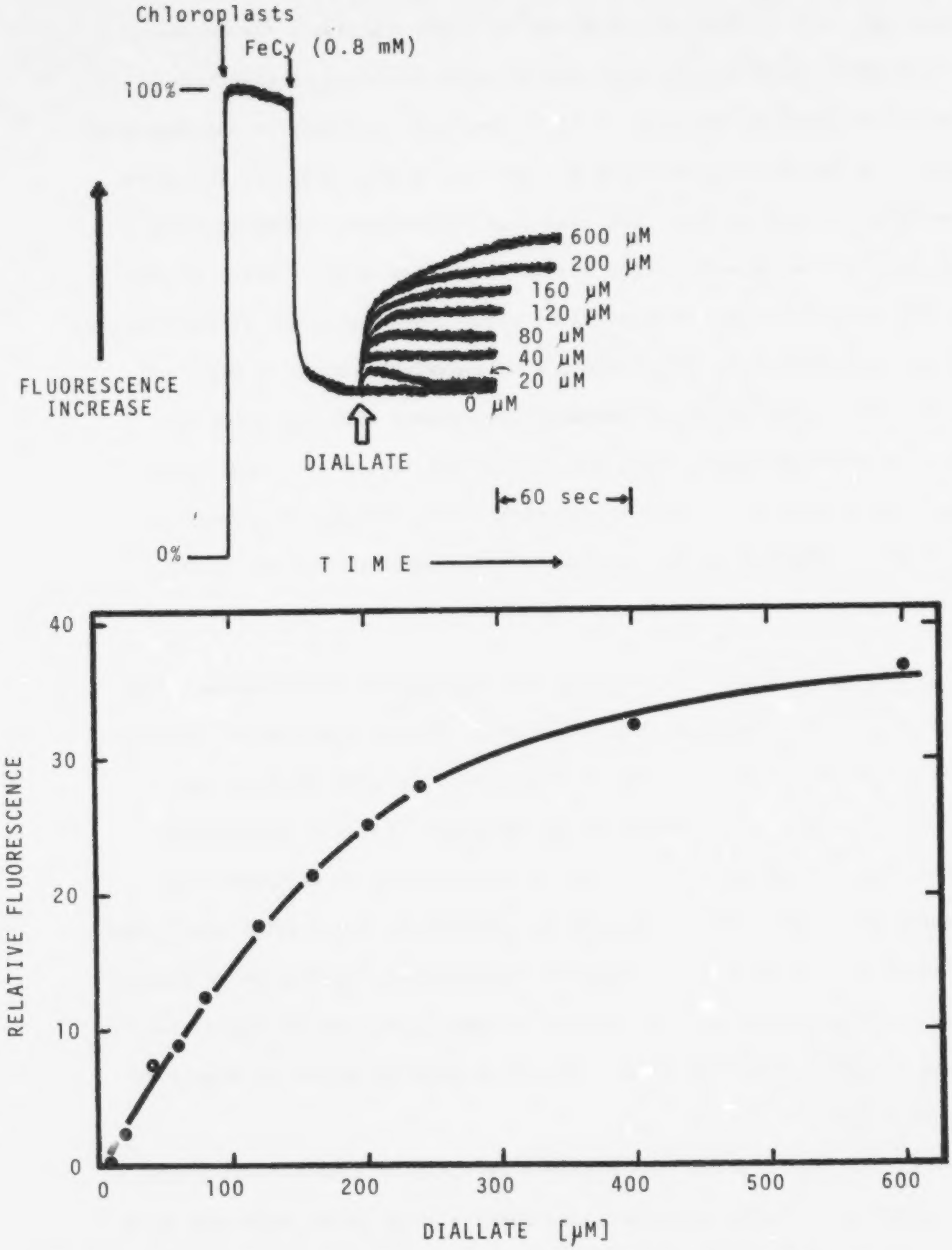


Fig. V-3. Effect of diallate on fluorescence of isolated spinach chloroplasts. A, tracings from recorder. B, maximal fluorescence at respective diallate concentrations.

I_{50} value of diallate effect on oxygen evolution was about 20 uM for the same $H_2O \longrightarrow$ Ferricyanide assay (see Table III-1 in the Final Report of the preceding year). The titer value obtained with the fluorescence technique is closer to the photosystem II assay ($H_2O \longrightarrow PD_{ox}$) employed during the preceding year (see Fig. III-5 in the Final Report of the preceding year).

Comparisons between the results obtained with the fluorescence technique and those obtained with the oxygen electrode assay bring about the following points: (a) with diallate the results from the chlorophyll fluorescence assay do not agree with those from photosynthetic electron transport studies, (b) fluorescence kinetics with diallate are not straight forward as those with diuron, and (c) similar to the conclusion of the preceding year, the fluorescence analysis strongly suggests possible multiple modes of action of this herbicide on photosynthetic reactions in chloroplasts. While this herbicide appears to be an interesting inhibitor of photosynthesis and may be useful in the mechanistic analysis of photoreactions of photosynthesis, it is highly elusive in our effort to pinpoint its biochemical mode of action.

Effects of Trifluralin. In order to examine trifluralin action, an oxygen electrode was inserted into the fluorometer cuvette, and both oxygen evolution and fluorescence were monitored simultaneously (Fig. V-4B). As for comparison the effect of diuron was also analyzed by the simultaneously measuring technique (Fig. V-4A). Diuron inhibits electron transport and stimulates chlorophyll fluorescence immediate after addition. Trifluralin, on the other hand, acts to decrease both fluorescence and oxygen evolution. In addition, the introduction of oxidized phenylene diamine (PD_{ox}) stimulates oxygen evolution without affecting fluorescence, and the last addition of diuron completely inhibits oxygen evolution allowing fluorescence to increase

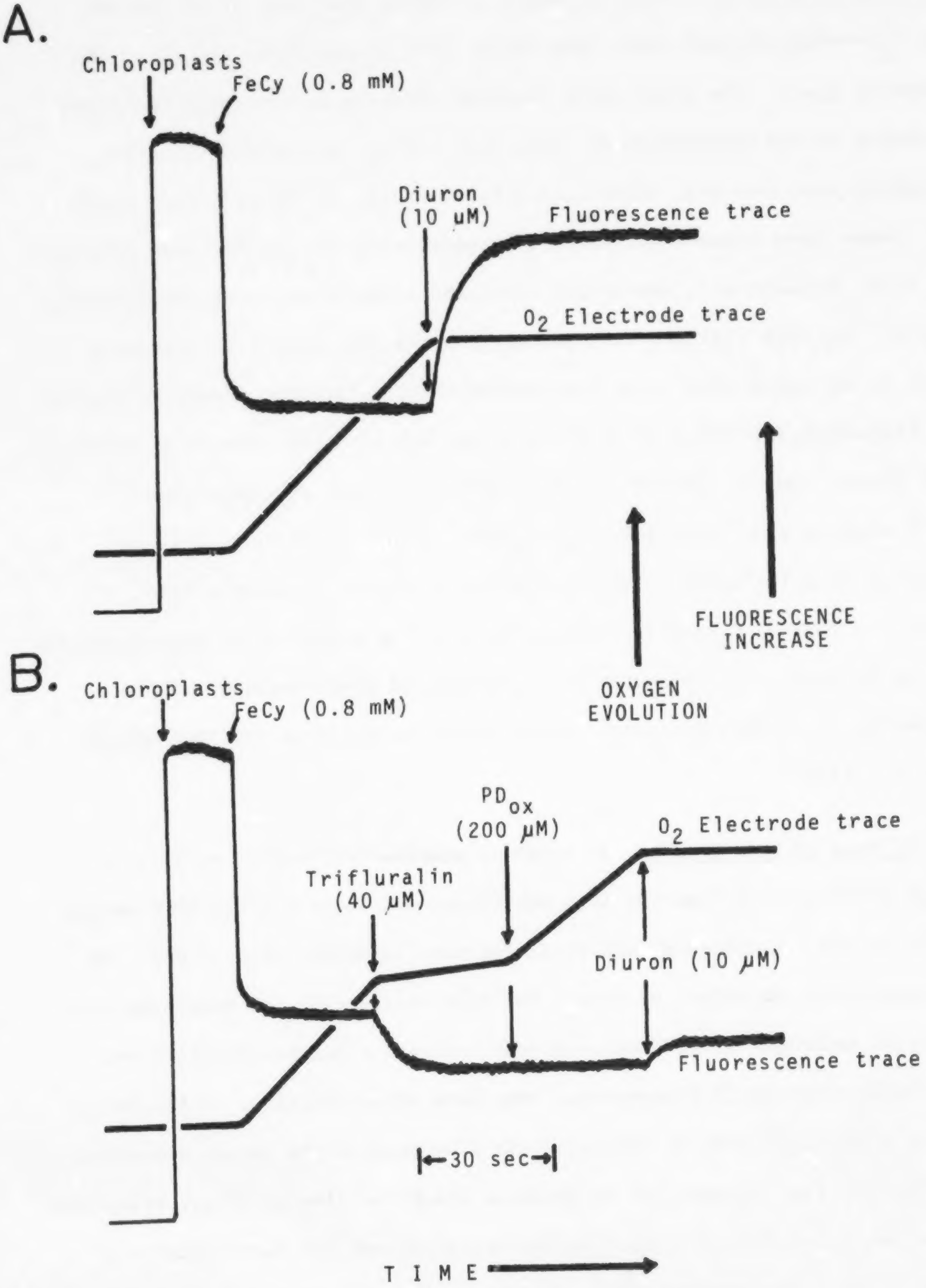


Fig. V-4. Effect of diuron (A) and trifluralin (B) on oxygen evolution and fluorescence by isolated spinach chloroplasts.

only slightly.

If this herbicide were to act between photosystems I and II as our work of the preceding year strongly suggested, trifluralin addition should have increased fluorescence in a manner similar to diuron addition, and a subsequent addition of PD_{ox} should have decreased fluorescence. The results obtained can best be interpreted in terms of chemical quenching of chlorophyll fluorescence by trifluralin in a manner similar to quinone inhibitors of photosynthetic electron transport. Although the data are not presented here, we have observed that this herbicide quenched fluorescence when added before ferricyanide, i.e., soon after chloroplast addition. This additional observation further supports the chemical quenching idea.

The ability of trifluralin to quench chlorophyll fluorescence was examined in the manner illustrated in Fig. V-5A. The titration curve in Fig. V-5B indicates that fluorescence decreases linearly to about 16 uM with a half-maximal effect obtained at about 10 uM. The linear decrease supports the idea of chemical quenching again. Nevertheless, the value for a half-maximal effect closely agree with the I_{50} value for the inhibition of oxygen inhibition (see CHAPTER III of the Final Report of the preceding year). Although the data in Fig. V-5B are not corrected for trifluralin absorption of blue light, the maximum degree of decrease in fluorescence is only about 15% of total fluorescence ascribable to chloroplasts; about 35% of chlorophyll fluorescence is apparently unaffected by this chemical.

EVALUATION OF THE CHLOROPHYLL FLUORESCENCE TECHNIQUE

The chlorophyll fluorescence technique is relatively simple and can provide data concerning whether a given herbicide is an inhibitor of photosynthetic electron flow or not. This point is well exemplified in the effect of diuron (Figs. V-2 and V-4). On the other hand, if a herbicide acts

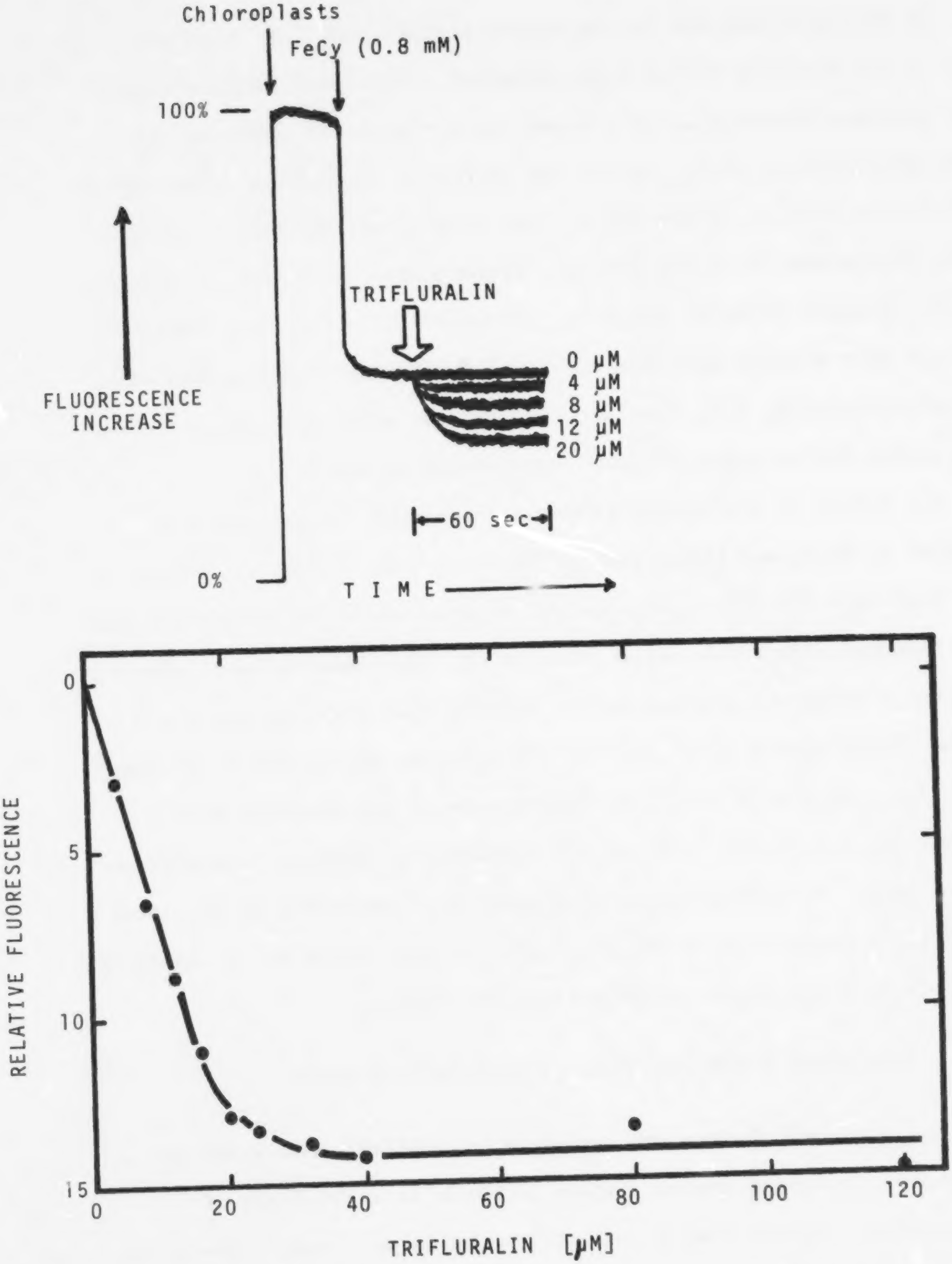


Fig. V-5. **Effect of trifluralin concentrations on fluorescence by spinach chloroplasts. A, recorder tracings. B, minimal fluorescence at respective trifluralin concentrations.**

in multiple mode as in the case of diallate (Fig. V-3) and particularly if it acts as a chemical quencher of chlorophyll fluorescence as in the case of trifluralin (Figs. V-4 and V-5), this technique is ineffective and cannot provide exact modes of action of herbicides. However, if it is used in conjunction with an oxygen electrode (Fig. V-4), this technique can complement the assessment of herbicidal phytotoxicity in photosynthetic electron transport.

In our leaf disk fluorescence assay, we employed a flow system in which a leaf disk was positioned stationary and a herbicide solution was circulated into the leaf disk compartment. The leaf disk was obviously treated in an unnatural condition to minimize possible noise in the measurement of fluorescence. In addition, to obtain optimal fluorescence responses, the plant must be pre-conditioned prior to preparation of leaf disks (see CHAPTER IV of the Final Report of the preceding year). Although a fluorescence device was considered to be added in the combined method used in the work of CHAPTER IV, we decided against this addition of a fluorescence device, mainly because the fluorescence device was not free of biological and instrumental problems.

CHAPTER VI

EFFECTS OF HERBICIDES ON ULTRASTRUCTURE OF ROOT TIP CELLS AND LEAF CELLS, WHEN GIVEN TO THE ROOT OF YOUNG SOYBEAN PLANTS

By Linda K. E. Graham and Robert J. Lowry

INTRODUCTION

The electron microscopic work of the preceding year (see CHAPTER VII of the Final Report of the preceding year) revealed that treatments of isolated corn root tips with herbicide solutions for 2 hours caused alterations of ultrastructures in the root tip cells. Alterations were found frequently in one or more of the following structures: mitochondria, plastids, spindle microtubules, and the orientation of the cell plate. Infiltration of herbicide solutions into soybean leaf segments induced little ultrastructural effects.

Induction of abnormal morphology of organelles can sometimes precede alterations of physiological parameters in root tips. The most notable example of this type was massive cell damage caused by an addition of 10^{-3} M 2,4-D or 2,4,5-T, which did not affect root tip respiration as drastically as the ultrastructural alterations. Herbicides which affected the morphology of spindle microtubules and cell wall formation in general caused much smaller effects on root respiration. Although from the electron microscopic studies alone possible biochemical modes of action of herbicides cannot be derived, ultrastructural studies certainly add another dimension in the nature of phytotoxicity of herbicides.

The studies performed during the preceding year with an electron microscope had at least two drawbacks: (i) herbicide application was made to isolated tissue pieces, an unnatural method of herbicide application, and (ii) the time involved in the preparation of thin sections for electron microscopy took more than 2 days. During the present year, we endeavored to rectify these drawbacks. We thus applied herbicides to the root solution of a young soybean plant, and tissues to be analyzed were subjected to a shorter method of dehydration and embedding.

MATERIALS AND METHODS

Young soybean plants were conditioned in a solution culture as described before. A herbicide was added to the root solution and pieces of trifoliolate leaves and secondary root tips were separated after two hours of herbicide treatment. These tissue pieces were subjected to the procedure for thin section preparation.

A shorter method of dehydration and embedding by Muller and Jacks (1975) was adopted for most of the work for the present year. Thin sections were prepared in the following manner:

a. Fixation of plant material with glutaraldehyde (2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2-7.4) for one hour at room temperature. The fixative was removed by washing with 3 changes of 0.05 M cacodylate buffer at pH 7.2-7.4, 5 minutes per each wash with agitation.

b. Post-fixation with osmium (2% osmium tetraoxide in 0.05 M cacodylate buffer at pH 7.2-7.4) for one hour at 0-4° C. The plant material was rinsed with three changes of cold cacodylate buffer, 15 minutes each with occasional agitation.

c. Dehydration of the fixed material with 2,2-dimethoxypropane (DMP). The fixed material was immersed, after thorough washing, in about

5 ml of acidified DMP (1 drop of concentrated HCl in 100 ml of DMP) for 15 minutes at room temperature. The plant material was transferred to DMP-acetone mixture (1:1) for 15 minutes at room temperature, and to absolute acetone for 15 minutes at room temperature twice.

d. Embedding. The dehydrated material was transferred to Spurr's low density epoxy resin in the following sequence: first to the Spurr's resin-acetone mixture (1:2) for about 20 minutes until the tissue sank, then to another mixture (1 resin : 1 acetone) until the tissue sank, and to the resin (no acetone), and finally to the Spurr's resin in gelatin capsules. The tissue was allowed to sink. The resin was polymerized at 70° C overnight.

e. Sectioning of the embedded tissue with a LKB ultramicrotome using a glass knife to about 60 nm thicknesses.

f. Staining for 30 minutes with 0.5% uranylacetate, followed by Reynold's lead citrate for 2 minutes.

The prepared sections were examined by a RCA or a Zeiss electron microscope. Microscopic observations were routinely carried out from the highest concentration of herbicide treatment (10^{-3} M) to the concentration at which no significant effect was observed.

RESULTS

A. EVALUATION OF THE RAPID METHOD OF THIN SECTION PREPARATION

The rapid chemical dehydration method of Muller and Jacks shortens the dehydration step considerably. Since shortening of any step in the preparation of thin sections would aid rapid observations of herbicidal effects, we employed their method and further attempted to shorten other preparatory steps. For example, the periods of fixation and washing were attempted to be shortened by introducing agitation during such steps. The use of epon resin embedding mixture of the preceding year was changed to the Spurr's low viscosity epoxy resin as suggested by Muller and Jacks (1975); this change was necessitated not only to quicken the time of preparation but by maintaining the quality of electron micrographs. The use of the last year's epon resin after quick dehydration of tissues by DMP resulted in poor quality preparations of thin sections.

Table VI-1 compares the new method with the previous method. With the new method, thin sections can be made in the second day and the examination of sections with an electron microscope can easily be done within 48 hours. In addition, as indicated by Muller and Jacks, ultrastructural features of subcellular structures in materials dehydrated by DMP were maintained in all respects. In some respect, the new method tended to give better contrasting structures, perhaps because of retention of components susceptible to extraction during the more prolonged exposure to aqueous alcohol or acetone in graded solvent replacement of the previous method.

B. HERBICIDAL EFFECTS ON THE ULTRASTRUCTURE OF ROOT TIP CELLS AND MESOPHYLL CELLS

While numerous electron micrographs have been examined, many of the ultrastructural alterations observed in soybean root tip cells and leaf cells were similar to those observed in the previous year's investigations. We have therefore chosen fewer numbers of electron photomicrographs to illustrate herbicidal effects. They are summarized in 30 photomicrographs and in Tables VI-2 and VI-3.

Table VI-1

Comparison of time consumption between the previous method and the method of Muller and Jacks for microscopic examination. The previous method refers to the method employed in the preceding year, and the method of Muller and Jacks to the method published in J. Histochem. Cytochem. Vol. 23, pp. 107-110 (1975).

Step	Previous Method	Method of Muller & Jacks
Glutaraldehyde Fixation	2 hours	1* hour
Washing	1	¼*
Osmium post-fixation	1½	1*
Washing	1	¼*
Dehydration	2½	1
Resin Infiltration	Overnight	1
Resin Polymerization	Overnight	8 (overnight)
Total time	about 2 days	1 day

* Hours saved in the first four steps are due to agitation.

Table VI-2

Summary of effects of 16 herbicides, applied to the root of intact soybean plant, on the ultrastructure of secondary soybean root tip cells.

Young soybean plants were treated with herbicide for about 2 hours. ..... denotes normal appearance at all concentrations tested. The concentrations at which effects were observed are given in parentheses. For simazine and trifluralin, the highest concentrations used were 10^{-4} M because of solubility problem. All other herbicides were tested at 10^{-3} M as their highest concentration applied.

Herbicide	Mitochondria	Plastids	Cell Wall	Mitotic Figure	Other Effects
Amitrole	.....	.....	.....	.....	.....
Atrazine	.....	.....	.....	No mitotic cells observed	.....
Bromacil	.....	.....	.....	No mitotic cells observed	.....
Cacodylate	.....	.....	.....	.....	Some fibrillar material in vacuoles (10^{-3} M)
Chlorpropham	Enlarged, ring-shaped profiles of mitochondria and/or plastids; some with much reduced matrix or stroma (10^{-3}, 10^{-4} M)		Fewer cortical microtubules (10^{-3} M)	No mitotic cells observed	.....
2,4-D	Massive general cell damage (10^{-3} M)				
Diallate	Enlarged, ring-shaped profiles of mitochondria and/or plastids (10^{-3}, 10^{-4} M)		Fewer cortical microtubules; many binucleate cells; no microtubules present during cell plate formation; malformed cell plates (10^{-3} M)		.....

Table VI-3 --- continued

Herbicide	Mitochondria	Plastids	Cell Wall	Mitotic Figure	Other Effects
Diquat	Elongated, lobed or branched; dark material collected around cristae (10^{-3} M)	.....	.....	No mitotic cells observed	Much enlarged vacuoles
Diuron	.....	.....	.....	.....	.....
DNOC	Ring-shaped profiles of mitochondria and plastids (10^{-3}, 10^{-4} M)		Aggregates of vesicles between cell wall & plasmalemma (10^{-3}, 10^{-4} M)	Irregularly shaped nuclei causing some cells to appear binucleate (10^{-3}, 10^{-4} M)	Ring-shaped golgi; highly vacuolated cells; long pieces of rough ER (10^{-3}, 10^{-4} M)
DSMA	.....	.....	.....	.....	.....
Karbutylate	.....	.....	.....	.....	Some fibrillar material in vacuoles (10^{-3} M)
Monuron	.....	.....	.....	.....	.....
Propham	.....	.....	.....	.....	Irregularly-shaped vacuoles containing dark stained globules (10^{-3} M)
Simazine	.....	.....	Incomplete cell plate formation; Y-shaped cell plates; microtubules appear morphologically normal but disoriented (10^{-4} M)		Fibrillar inclusions (10^{-4} M)
Trifluralin	Ring-shaped profiles of mitochondria and plastids (10^{-4} M)		Fewer cortical microtubules (10^{-4} M)	Cell plate absent; binucleate cells (10^{-4} M)	Dark-staining cytoplasmic inclusions; fibrillar inclusion (10^{-4} M)

Table VI-3

Summary of effects of 16 herbicides, applied to the root, on the ultrastructure of soybean leaf cells. Young soybean plants were treated with herbicides for about 2 hours.

Herbicide	Observed Effect on Ultrastructure
Amitrole	No significant effect observed at 10^{-3} M.
Atrazine	No significant effect observed at 10^{-3} M.
Bromacil	No significant effect observed at 10^{-3} M.
Cacodylate	No significant effect observed at 10^{-3} M.
Chlorpropham	No significant effect observed at 10^{-3} M.
2,4-D	Some myeline-like figures observed in vacuoles, but cells appear normal at 10^{-3} M.
Diallate	No significant effect observed at 10^{-3} M.
Diquat	No significant effect observed at 10^{-3} M.
Diuron	No significant effect observed at 10^{-3} M.
DNOC	No significant effect observed at 10^{-3} M.
DSMA	No significant effect observed at 10^{-3} M.
Karbutylate	No significant effect observed at 10^{-3} M.
Monuron	No significant effect observed at 10^{-3} M.
Propham	No significant effect observed at 10^{-3} M.
Simazine	Mitochondria appear swollen at 10^{-4} M; 10^{-3} M not tested because of solubility problem.
Trifluralin	No significant effect observed at 10^{-4} M; 10^{-3} M not tested because of solubility problem.

The survey of the two tables indicates that herbicides applied to the root system of intact soybean plants caused marked effects on the ultrastructure of the root tip cells (Table VI-2), but the morphology of leaf cells was not much affected (Table VI-3).

Amitrole, cacodylate, diuron, DSMA, karbutylate, monuron, and propham caused very little effect on the ultrastructure of root tip cells; any effect observable at the highest concentration examined was only restricted to somewhat different inclusions in the vauoles without affecting the rest of the ultrastructures of the cytoplasm (Figs. VI-4, VI-9, VI-10, VI-11, VI-15, VI-17). All other herbicides gave one of more of the following effects on the morphology of root tip cells: abnormal morphology of organelles (mitochondria, plastids, golgi, and nuclei), the number and orientation of spindle microtubules and cell plates, some inclusions in the space between the plasmalemma and the cell wall, and abnormal vacuolation (Table VI-2).

The ultrastructure of mitochondria and plastids in the root tip cell can be so drastically altered by chlorpropham, diallate, diquat, DNOC, or trifluralin that it becomes difficult to distinguish which organelles (mitochondria or plastids or both) were deformed (Figs. VI-1, VI-3, VI-19, VI-20, VI-21, VI-25). Diquat at 10^{-3} M frequently deformed normally ovoid mitochondria into oblong, lobed, and branched forms (Fig. VI-28). When the morphology of golgi bodies was affected, it assumed a ring-shape (Fig. VI-3). DNOC acted to alter the shape of nucleus (Fig. VI-2), to accumulate vesicular materials between the cell wall and the plasmalemma (Fig. VI-3), and to maintain abnormally long rough endoplasmic reticulums (Figs. VI-1, VI-3).

Mitotic processes were affected by several herbicides. With treatment with atrazine, bromacil, chlorpropham, or diquat, no mitotic figures were observed (e.g., Fig. VI-16). With treatment with trifluralin, spindle microtubules are apparently not formed and binucleated cells occur (Fig. VI-13, VI-14). Diallate effect is apparently not as complete as trifluralin in dissolving spindle microtubules; diallate-treated root tips contained cells with two nuclei as well as cells with half-formed or malformed cell plates with very few or no spindle microtubules transversing the forming cell plates (Figs. VI-23, VI-24, VI-25, VI-26). Simazine acted to disorient microtubules, causing a formation of branched or irregularly formed cell plates (Figs. VI-5, VI-6, VI-7, VI-8).

Perhaps the most drastic effect on cellular morphology is induced by treatment with 2,4-D; it causes massive, general damage of root tip cells (Fig. VI-12). Herbicides such as diquat and DNOC caused excessive vacuolation (Figs. VI-1, VI-9).

Very little evidence for ultrastructural alteration was observed in soybean leaf cells from plants treated with herbicide for 2 hours at the roots (Table VI-3 and Fig. VI-29). Mitochondria were frequently somewhat swollen, but the frequency of this response suggests that the effects might be due to vacuum infiltration of fixative. No evidence of chloroplast disturbance was noted throughout the present study. The two-hour treatment time was probably not long enough for accummulation of herbicide in the leaves in quantities sufficient to cause ultrastructural alterations.

Since undifferentiated, meristematic cells appear to be more readily influenced by herbicide treatments (CHAPTER VII of the Final Report of the preceding year), we attempted to examine the cells in the apical meristem of young soybean plants whose roots were treated with a herbicide for 2 hours. Figure VI-18 shows a control meristematic cell of the soybean shoot apex. The organelles appear similar to those in the root meristem. However, mitotic cells were difficult to observe and probably not common enough for easy assessment of herbicide effects on mitosis and cell plate development. In addition, the meristem itself was difficult to locate because it is obscured by leaf primordia. Although the meristems of soybean plants treated with a few herbicides were examined, no significantly detectable effect was observed. Similar to the lack of effect on mesophyll cells by herbicides, we concluded that a given herbicide was not accummulated in large enough quantities to cause any ultrastructural alterations in shoot meristematic cells. From these observations, we discontinued our analysis of shoot meristematic cells.

DISCUSSION

The rapid dehydration and embedding procedure of Muller and Jacks roughly halves the time required for preparation of thin sections for electron microscopic examinations (Table VI-1), preserves all ultrastructural features of the cell, and produces photomicrographs equally good as or perhaps in a little more contrasting manner than the conventional method used in the study of the preceding year. The rapid method is reliable and should be useful when large numbers of specimens are to be processed and when the examination of herbicidal effects on cellular ultrastructures must be accomplished in 48 hours.

Despite the difference in the method of herbicide application between the studies of the two successive years (force feeding of herbicide for the study of the preceding year vs. a more natural means of application this year) and also the difference in plant materials used (corn root tips of the preceding year vs. soybean root tips of this year), it is remarkable to note that (a) herbicides are more effective in causing ultrastructural alterations of root tip cells than those of mesophyll cells, and (b) herbicidal effects are primarily noted in the ultrastructure of organelles and mitotic activities of root meristematic cells. Obviously, the different methods of herbicide application brought about different effects; (a) several, but not all, herbicides caused more readily recognizable damage in the ultrastructure in the cells of herbicide-infiltrated leaves than in that of leaf cells of soybean plants to whose roots herbicides were applied, and (b) with exceptions of a few herbicides, herbicides caused somewhat different effects to the root tip cells. The latter difference can be examined in more detail. Herbicides, such as amitrole, atrazine, bromacil, cacodylate, diuron, DSMA, karbutylate, minuron, and propham, exerted less drastic effects in the root tip cells of this year. Some other herbicides, such as chlorpropham, diallate, diquat and DNOC gave more pronounced effects this year than the preceding year. The effects of 2,4-D, simazine, and trifluralin are more or less identical in their action.

These comparisons of the results from the studies of two successive years lead us to the following conclusions: (i) root meristematic cells are more sensitive to herbicides than mesophyll cells, (ii) forced entry of herbicides induces faster responses, and (iii) significant differences observed by two different roots (corn root tips of the preceding year vs. soybean root tips of this year) may indicate species specificity in response to herbicidal treatment.

It is of interest to note that some herbicides (e.g., atrazine, bromacil, DNOC, karbutylate, and simazine) caused inhibition of mesophyll photosynthesis within 1 hour after their application to the root solution at 10^{-3} or 10^{-4} M (Table II-3), while their effect was not detected at all ultrastructurally in leaf cells (Table VI-3). On the other hand, 2,4-D and trifluralin caused more pronounced effects on the morphology of root tip cells in

2 hours, but they inhibited root respiration to a much less extent than their effects on root cell ultrastructure after the same period of treatment. In order to make any early assessment of herbicidal effects, the data from both physiological and morphological examinations are highly valuable.

In order to detect the more direct action of herbicides, we chose a short treatment period of two hours in most studies of both the preceding and the present years, and herbicidal concentrations as high as 10^{-3} and in some cases 10^{-2} M have been employed. In many instances, the degree of herbicidal effects is dependent upon the concentrations used and the faster effect appears at highest concentration. With respect to ultrastructural alterations, an emphasis in the literature has been placed on the examination of mesophyll chloroplasts after much longer periods of herbicide treatment (see Anderson and Thomson, 1973; Ashton and Crafts, 1973). Judging from our results of both years, the cellular ultrastructures of root tip cells are more readily altered by herbicides and at least in some cases such alterations may cause secondary effects on mesophyll cells.

EVALUATION OF METHOD

The rapid hydration and embedding method of Muller and Jacks allows the examination of thin sections with an electron microscope in 48 hours. The resultant electron photomicrographs are of high quality. It is highly recommendable for herbicide screening work.

Since herbicides were applied in a more natural manner this year than the preceding year and their effects were qualitatively very similar between the two year's investigations, we recommend the method of herbicide application used for the work of this year strongly. In addition, the information derived from the ultrastructural studies supplements and complements the results from physiological studies.

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Muller, L. L. and T. J. Jacks. 1975. Rapid chemical dehydration of samples for electron microscopic examinations. J. Histochem. Cytochem. 23: 107-110.

LEGENDS FOR ELECTRON PHOTOMICROGRAPHS

Fig. VI-1. Effect of 10^{-3} M DNOC on the morphology of mitochondria and plastids in secondary root tip cells of soybean. Note large, irregular vacuoles and extensive endoplasmic reticulum. X 5,200.

Fig. VI-2. Effect of 10^{-3} M DNOC on the morphology of nucleus in a secondary root tip cell of soybean. X 9,000.

Fig. VI-3. Effect of 10^{-3} M DNOC on the ultrastructures of mitochondria, plastids, golgi, and cell wall in secondary root tip cells of soybean. X 25,000.

Fig. VI-4. Control (untreated) soybean root tip cells processed for electron microscopy using the rapid dehydration and embedding method of Muller and Jacks (1975). X 5,100.

Fig. VI-5. Abnormal cell plate formation in secondary root tips treated with 10^{-4} M simazine. X 6,800.

Fig. VI-6. An enlargement of the area between the nuclei of Fig. VI-5. X 36,700.

Fig. VI-7. Abnormal cell plate formation in secondary root tips treated with 10^{-4} M simazine. X 34,000.

Fig. VI-8. Gaps in newly formed cell wall in a secondary root tip of soybean treated with 10^{-4} M simazine. Note the membrane accumulations. X 41,000.

Fig. VI-9. Effect of 10^{-3} M karbutylate on the cellular ultrastructure of a secondary root tip of soybean. Note large accumulations of dark material in vacuoles. X 5,700.

Fig. VI-10 Effect of 10^{-3} M monuron on the ultrastructure of secondary root tip cells of soybean. Normal appearance. X 5,000.

Fig. VI-11. Effect of 10^{-3} M propham on the ultrastructure of secondary root tip cells of soybean. Note granular material in vacuoles; otherwise the cells appear normal. X 5,700.

Fig. VI-12. Extensive cell damage in soybean root tip cells after treatment with 10^{-3} M 2,4-D. X 5,400.

Fig. VI-13. Effect of 10^{-4} M trifluralin on the ultrastructure of secondary soybean root tip cells. Note no microtubules between two nuclei. X 28,600.

Fig. VI-14. Formation of binucleate cell in secondary root tip cells of soybean after treatment with 10^{-4} M trifluralin. X 5,400.

Fig. VI-15. Effect of 10^{-3} M amitrole on the ultrastructure of secondary root tip cells of soybean. Normally appearing cells, except slightly enlarged vacuoles with dark stained material. X 4,700.

Fig. VI-16. Effect of 10^{-3} M atrazine on the ultrastructure of secondary root tip cells of soybean. Normally appearing cells, except slightly enlarged vacuoles with dark stained material. X 4,600.

Fig. VI-17. Effect of 10^{-3} M DSMA on the morphology of secondary root cells of soybean. Normally appearing cells. X4,300.

Fig. VI-18. Cells in the shoot apical meristematic region of soybean plant. X 11,700.

Fig. VI-19. Abnormally appearing mitochondria and/or plastids in secondary root tip cells of soybean treated with 10^{-3} M chlorpropham. X 6,500.

Fig. VI-20. Abnormally appearing mitochondria and/or plastids in secondary root tip cells of soybean treated with 10^{-3} M chlorpropham. X 20,700.

Fig. VI-21. An abnormally appearing mitochondrion or plastid in secondary root tip cell of soybean treated with 10^{-3} M chlorpropham. X 14,900.

Fig. VI-22. Normally appearing cells in secondary soybean root tips treated with 10^{-4} M chlorpropham. X 6,100

Fig. VI-23. Binucleate cells with an aborted cell plate in a secondary root tip of soybean treated with 10^{-3} M diallate. X 2,100.

Fig. VI-24. An enlargement of the area between two nuclei of a binucelate cell without spindle microtubules in a secondary root tip of soybean treated with 10^{-3} M diallate. X 23,000.

Fig. VI-25. Effect of 10^{-3} M diallate on the morphology of plastids in secondary root tip cells of soybean. Note the swollen plastids containing concentric membrane aggregations. X 16,000.

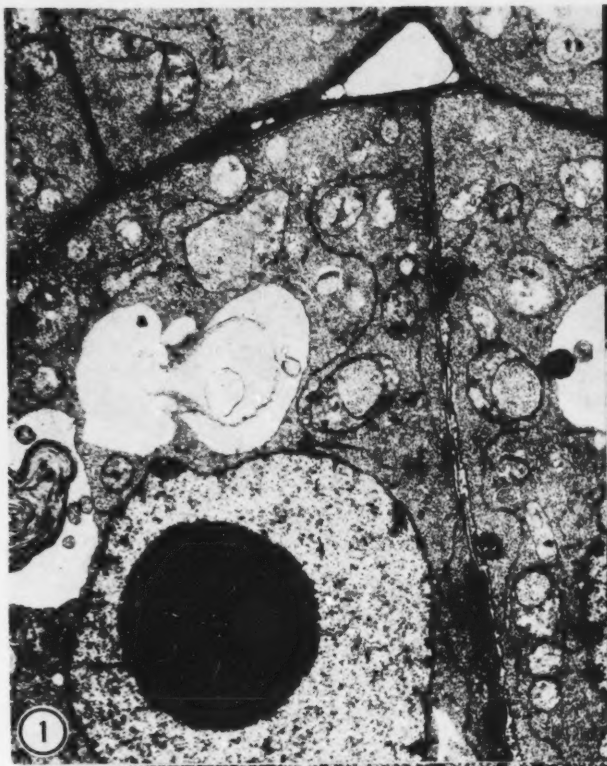
Fig. VI-26. Effect of 10^{-4} M diallate on the morphology of soybean secondary root tip cells. Normal appearance of cells. X 4,800.

Fig. VI-27. Enlarged vacuoles in secondary root tip cells of soybean treated with 10^{-3} M diquat. X 1,800.

Fig. VI-28. Enlarged, lobed, and branched mitochondria in secondary root tip cells of soybean treated with 10^{-3} M diquat. X 11,100.

Fig. VI-29. Leaf mesophyll cells of soybean treated with 10^{-3} M diquat. Cells appear normal. X 4,600.

Fig. VI-30. Effect of 10^{-4} M diquat on the morphology of secondary root tip cells of soybean. Somewhat enlarged vacuole with dark staining material. X 6,800.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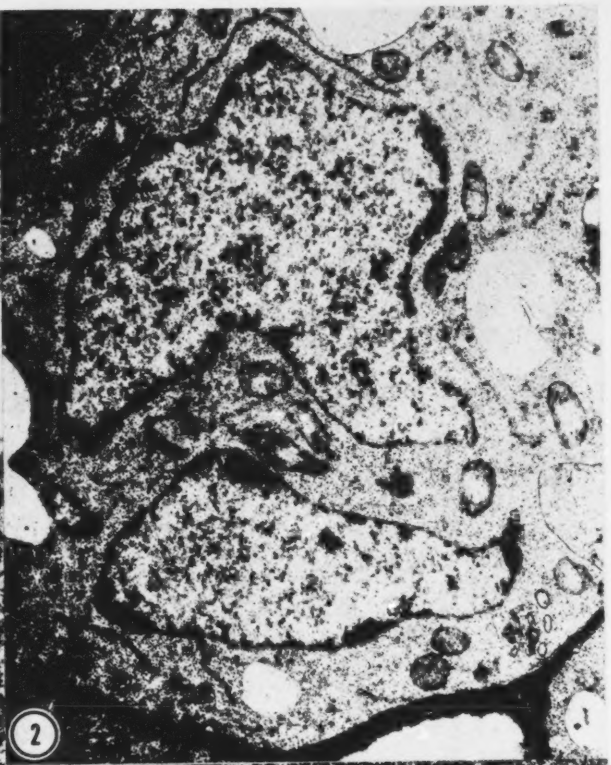
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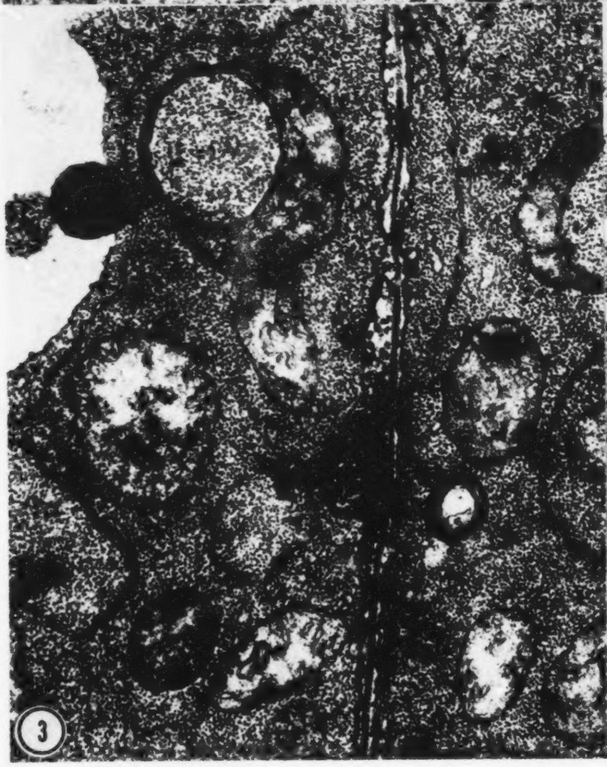
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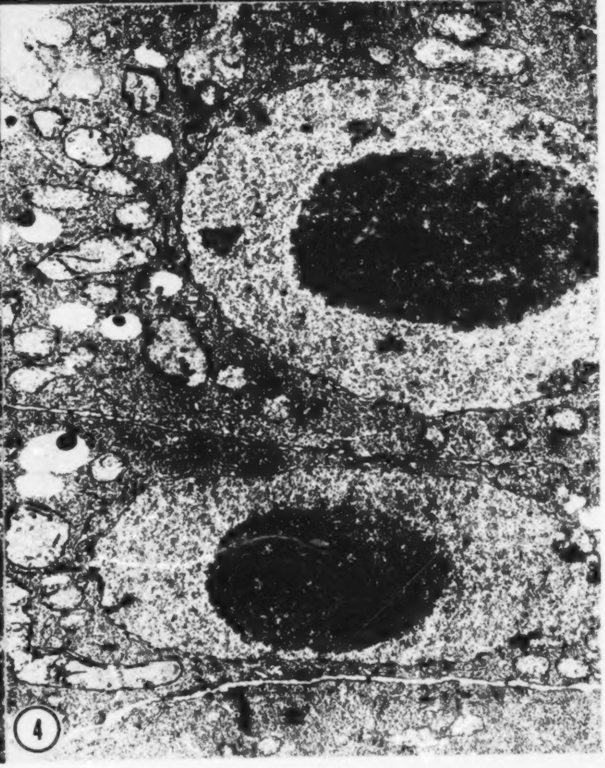
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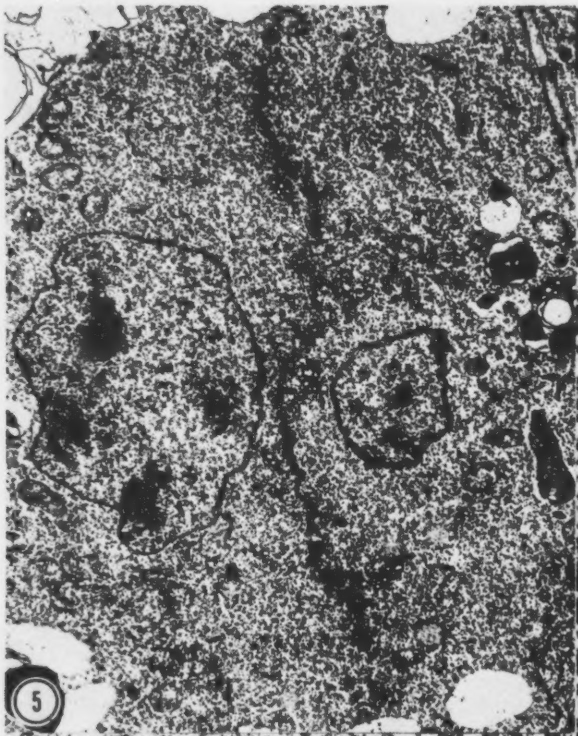
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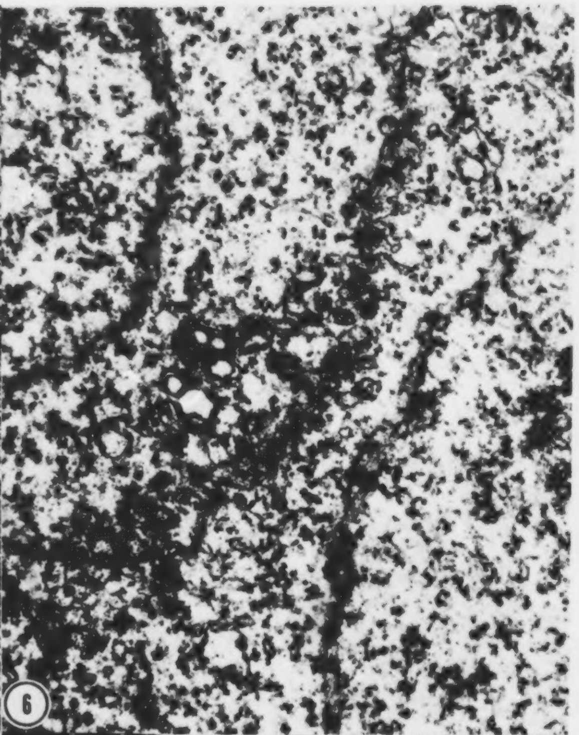
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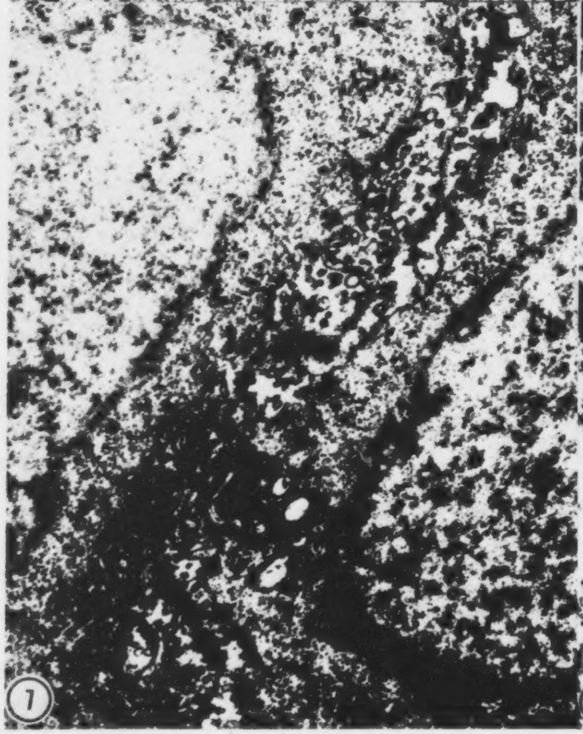
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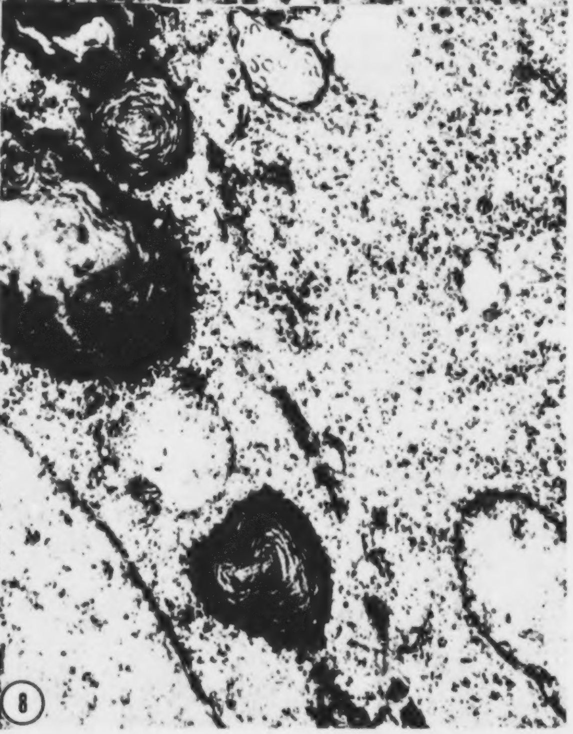
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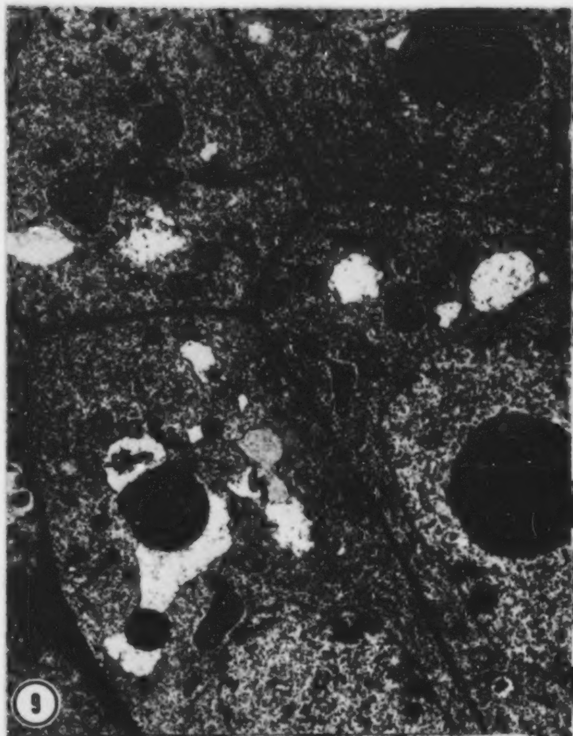

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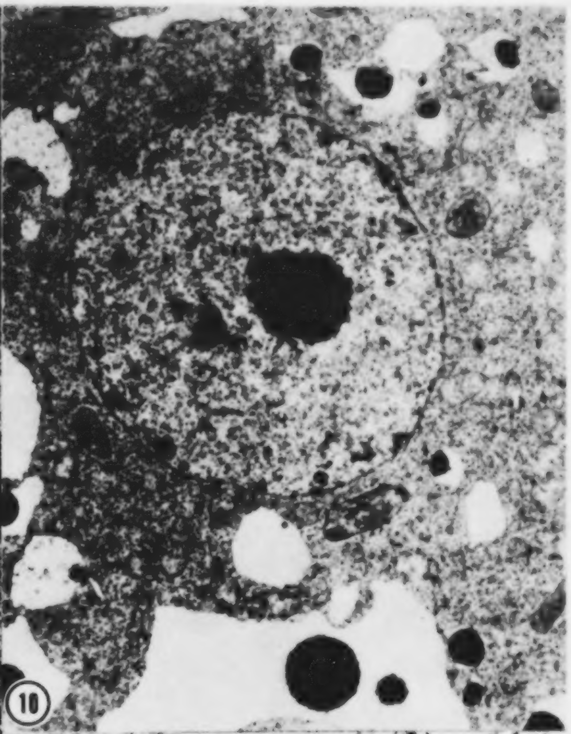

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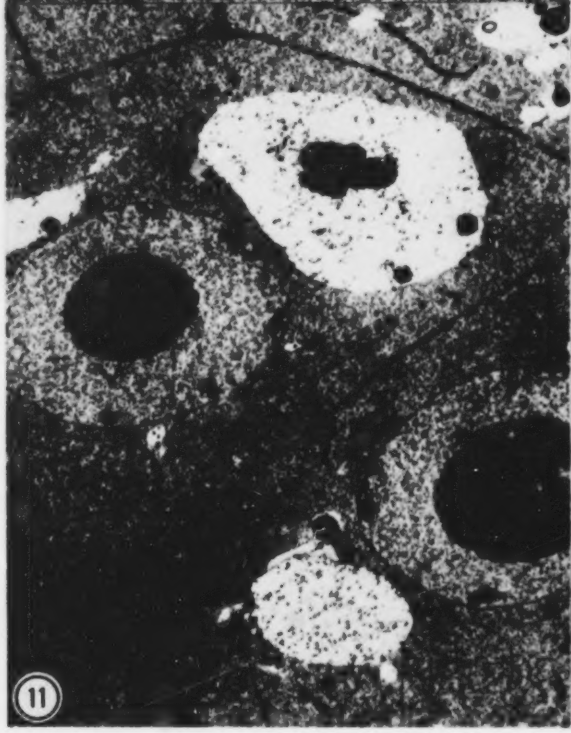

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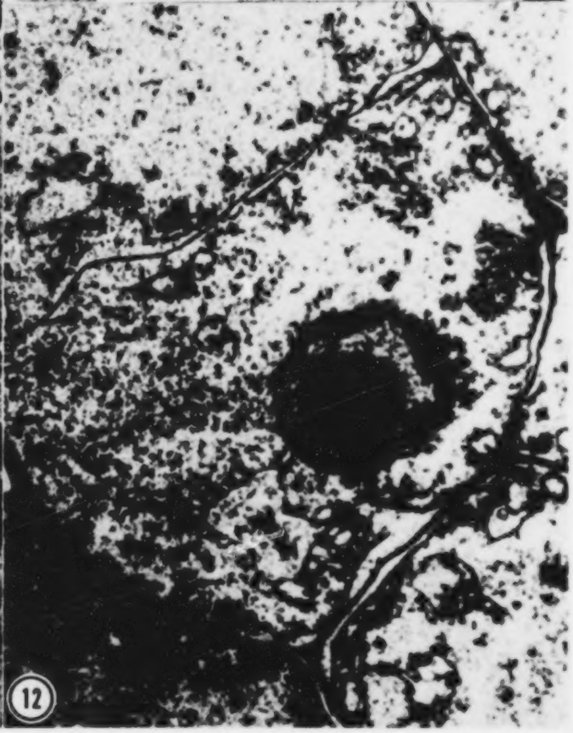

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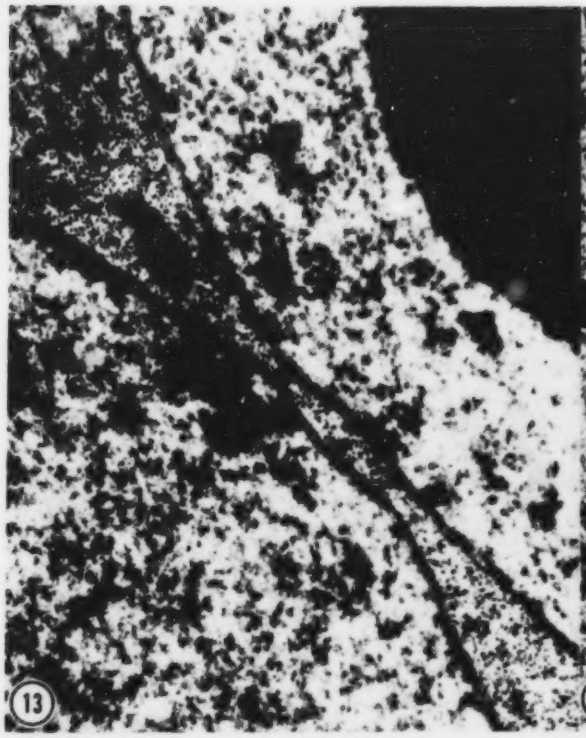

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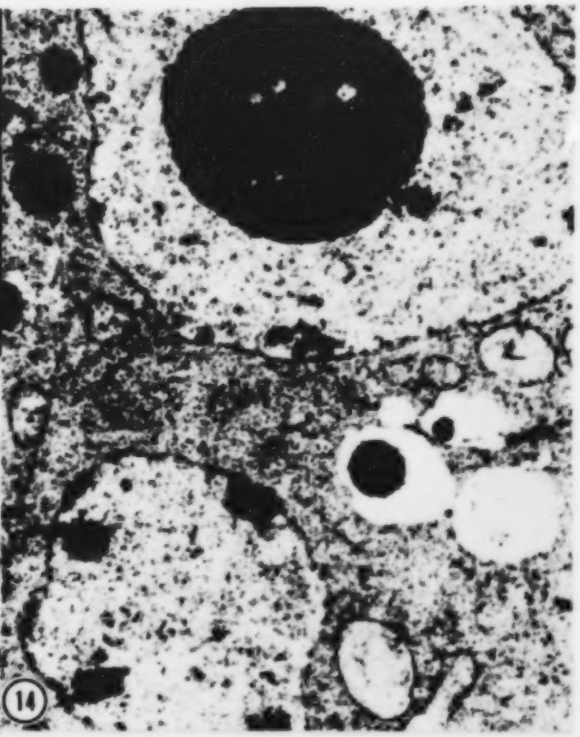

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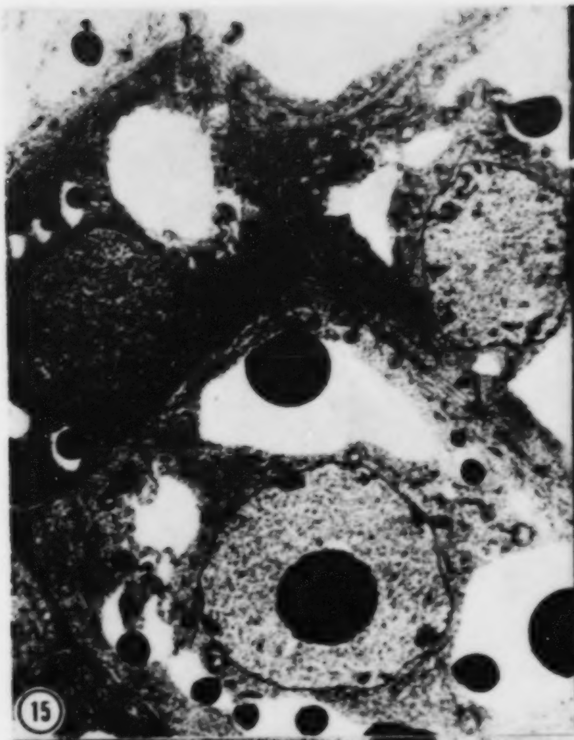
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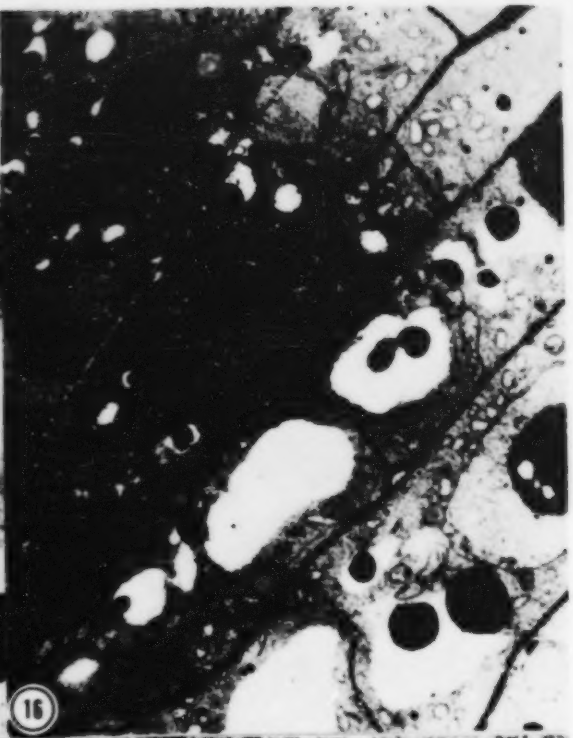
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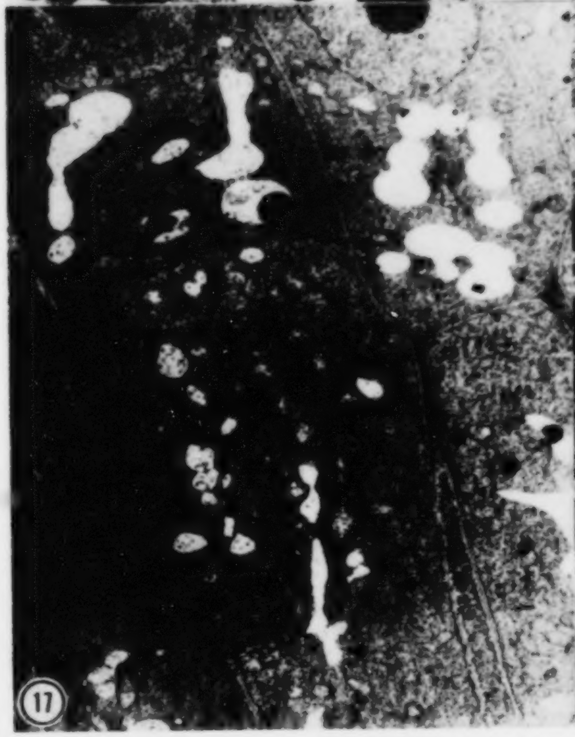
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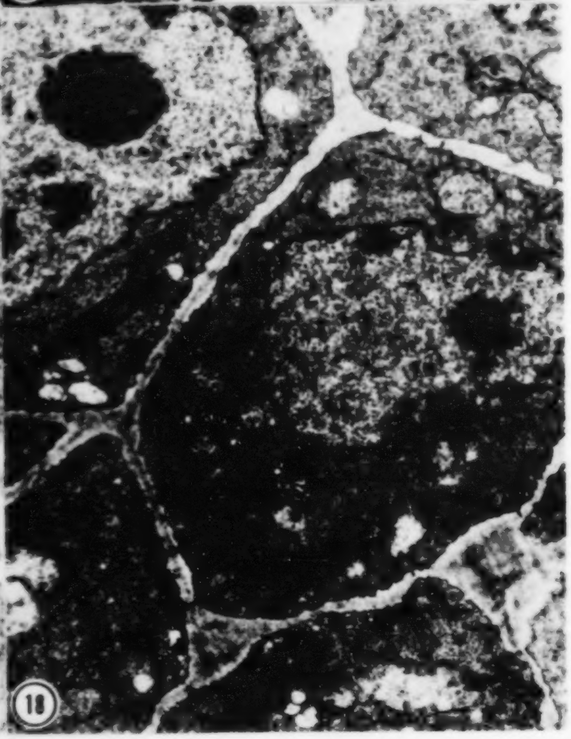
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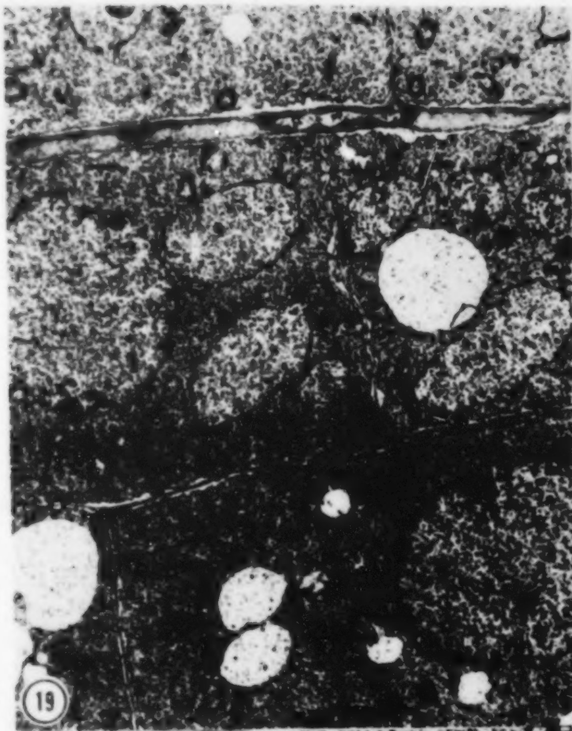

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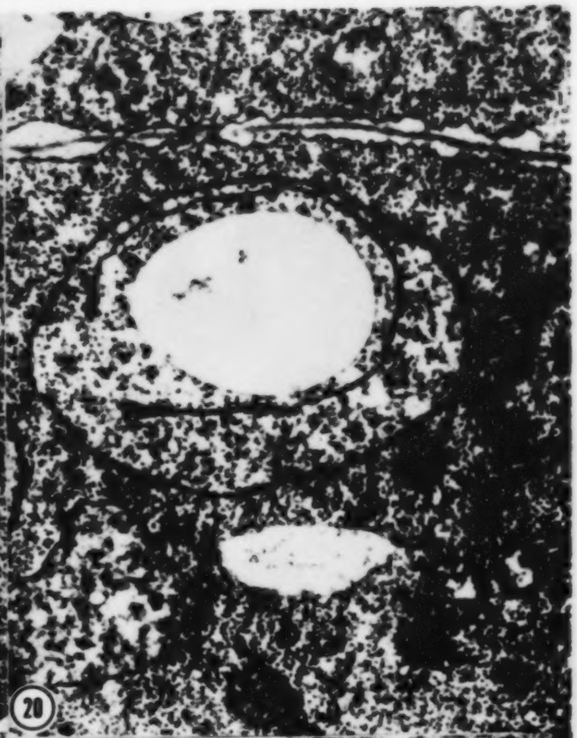

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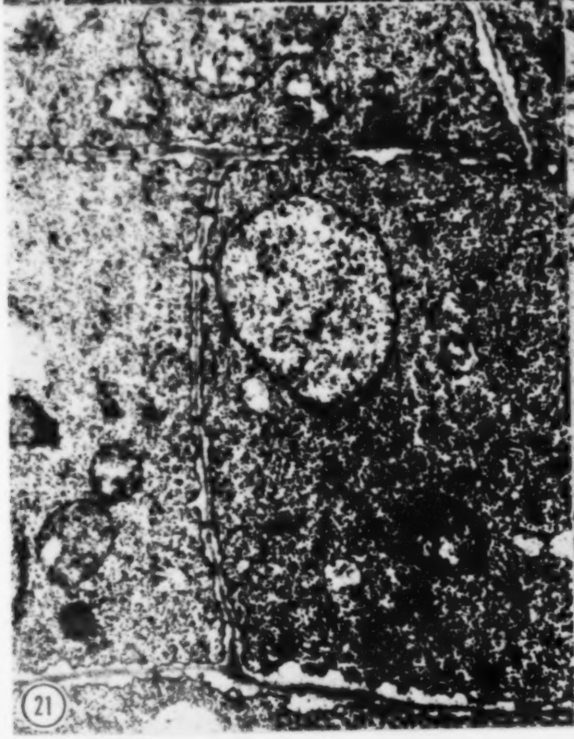

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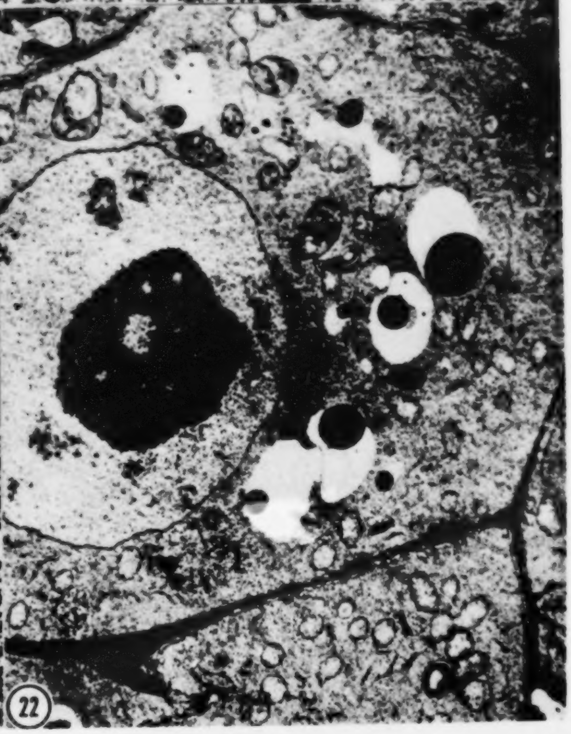

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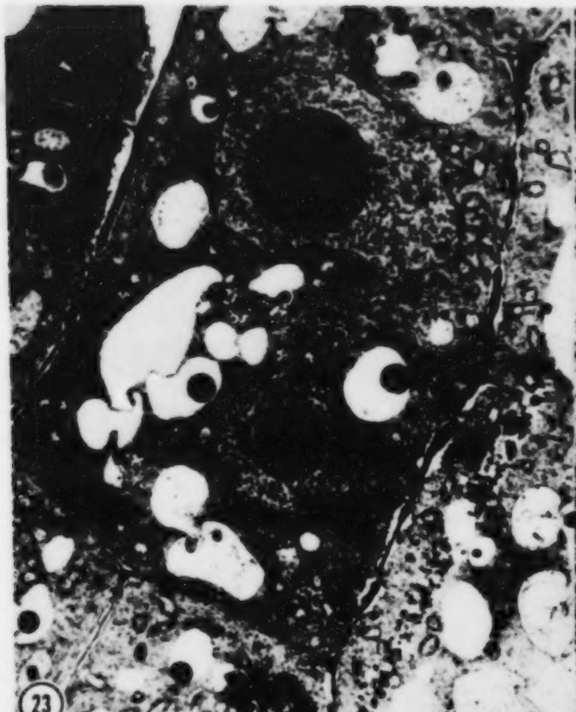
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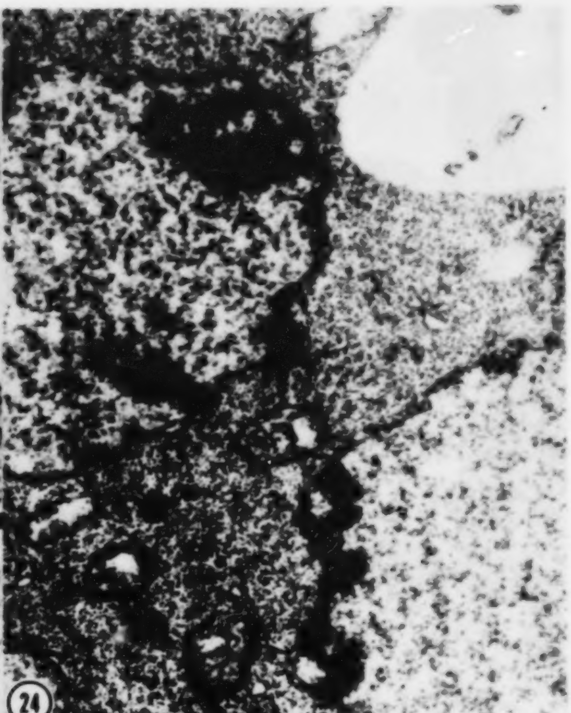
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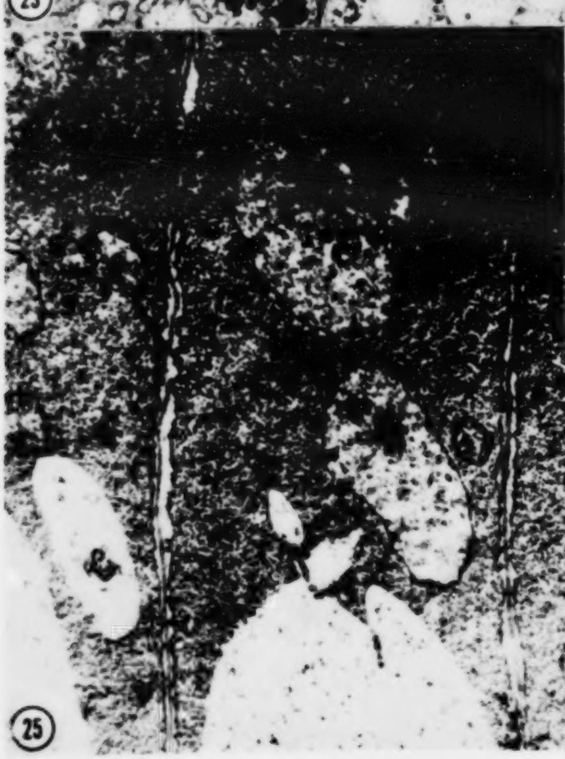
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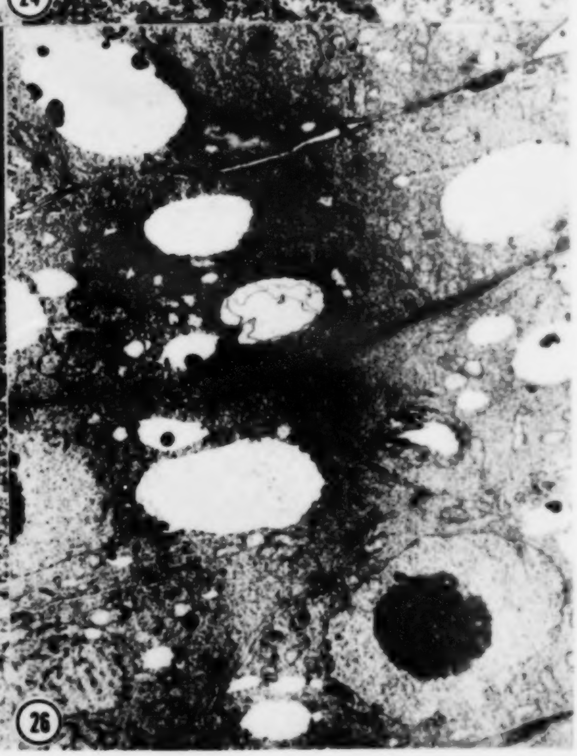
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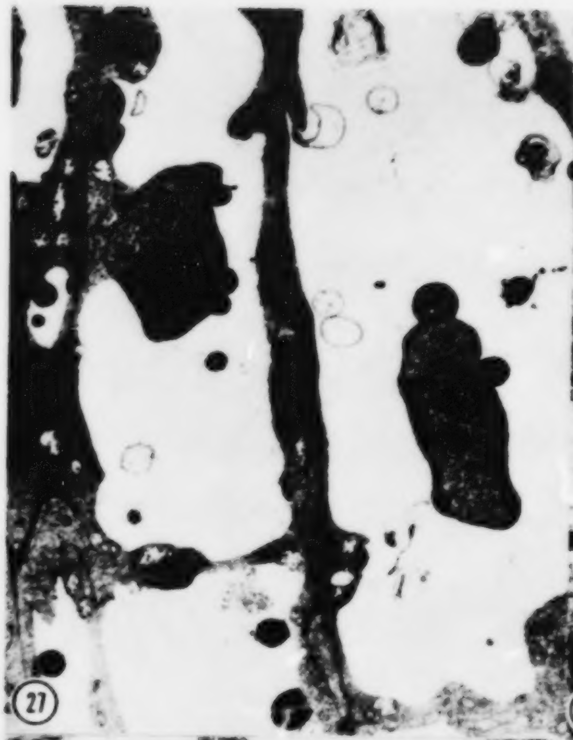
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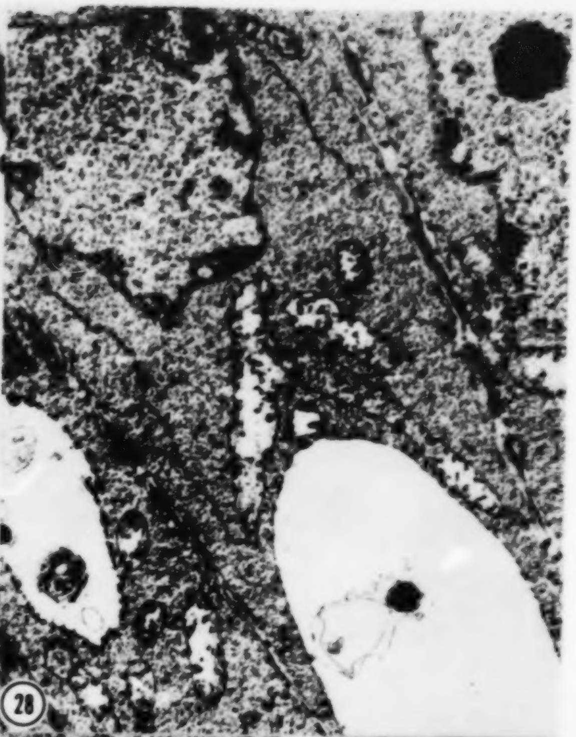
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CHAPTER VII

EXAMINATION OF ORGANELLES IN HERBICIDE-TREATED TISSUES

By James A. Guikema, Steven J. Robinson, Chin-Yu Chen, and Hiroshi Ikuma

INTRODUCTION

Electron microscopic observations of the preceding and the present years have pointed out that several herbicides act to alter the morphology of mitochondria and plastids in the root tip cells and the ultrastructure of chloroplasts in soybean leaf pieces to which herbicides were infiltrated (CHAPTER VII of the Final Report of the preceding year; CHAPTER VI of this report). While not all herbicides which affected cellular ultrastructures were effective in inhibiting the respiratory activity of the root and not all herbicides which inhibited photosynthesis of chloroplasts did not cause ultrastructural alterations in mesophyll chloroplasts, a question arises as to whether morphologically altered organelles would carry out the same biochemical activities as the regular, unaltered organelles. In order to answer this question, the current investigations were initiated.

For this purpose, mitochondria were isolated from soybean root segments which had been treated with a herbicide, and chloroplasts were isolated from soybean leaf pieces which had been treated with a herbicide. Initially these investigations were aimed at separating 'abnormal' from 'normal' organelles by means of a density gradient centrifugation technique, upon an assumption that deformed mitochondria or chloroplasts would have a bouyant density different from the regular, unaltered organelles. Judging from the results by Douce et al. (1972), such an assumption would be valid. As will be seen later, this approach caused unexpected difficulties; i.e., the so-called regular fraction, obtained from our regular differential centrifugation technique, consisted of several populations of organelles, and the composition of these populations varied from preparation to preparation. In other words, our mitochondria were much more heterogeneous than those of Douce et al's (1972). This situation posed a difficulty to analyze the differences in biochemical properties of regular and deformed organelles. In many of our experiments reported herein, we thus examined apparent biochemical differences between the organelles from herbicide-treated tissues and those from untreated tissues.

During the course of these investigations, it occurred to us that isolated organelles might change their ultrastructural features in response to herbicidal treatment _in vitro_. To test such a possibility, we examined the effect of diallate on isolated mung bean mitochondrial ultrastructure. We chose diallate, because, of the three herbicides which exerted their inhibition of mitochondrial activities at low concentrations, diallate was shown to bind to mitochondria irreversibly and stoichiometrically (CHAPTER II of the Final Report of the preceding year). The inhibition of mitochondrial function by the other two herbicides was removed by repeated washings of the mitochondria. Another aspect of interest in diallate was in the fact that this herbicide exerted multiple modes of inhibition of mitochondrial activities; not only electron transport but also ATPase and soluble malate dehydrogenase activities were inhibited by diallate.

Three types of experiments were thus performed: (A) analysis of the respiratory property of mitochondria isolated from soybean roots which had been treated with herbicide; (B) analysis of the photosynthetic activity of chloroplasts isolated from soybean leaves which had been treated with herbicide; and (C) ultrastructural analysis of isolated mung bean mitochondria treated _in vitro_ with diallate.

MATERIALS AND METHODS

Isolation of Mitochondria from Soybean Roots. Soybean seeds were soaked in a 5% (v/v) commercial Clorox solution for 15 minutes, rinsed thoroughly with tap water, imbibed in tap water overnight, and germinated on perforated aluminum sheets in 8" x 12" x 2½" planting trays for 7 days at 26° C in the dark. Roots were harvested from the germinated seedlings, rinsed with cold distilled water with several changes, and incubated in a 500 ml beaker with a treatment solution (distilled water or a herbicide solution) for 2 hours with aeration (air bubbling). About 100 gram roots were homogenized by hand in a porcelain mortar with a pestle with 500 ml of the extraction medium consisting of 0.5 M mannitol, 0.01 M phosphate buffer, pH 7.2, 0.005 M cysteine, 0.001 M EDTA, and 0.2% bovine serum albumin.

A crude mitochondrial fraction was obtained by differential centrifugation between 500 x g and 13,000 x g. The pellet was uniformly suspended in 1 ml of the suspending medium (0.5 M mannitol, 0.01 M phosphate buffer, pH 7.2, and 0.2% bovine serum albumin), and the whole suspension was placed on top of a discontinuous sorbitol gradient composed of 4.00 (bottom), 3.35 M, 2.85 M, 2.35 M, 1.85 M, 1.35 M, and 1.00 M (top), each with 1.7 ml, in a 12 ml heavy wall glass centrifuge tube (Sorvall). The tube was spun in a HB-4 swinging bucket rotor at 7,500 rpm (ca. 9,000 x g) for 45 minutes in a Sorvall RC-5 refrigerated centrifuge. It should be mentioned that we used sorbitol instead of sucrose in gradient (cf. Douce et al., 1972) because sorbitol was found a superior osmoticum in our preliminary study.

When 'purified' mitochondria were used, the crude mitochondrial fraction was spun successively to obtain a fraction between 500 x g and 5,500 x g.

Isolation of Chloroplasts from Soybean Leaves. Several mature leaves were removed from 8-10 week old soybean plants with two fully expanded trifoliolate leaves. They were cut into small pieces and a sample of approximately 5 gram was used for herbicide treatment and subsequent isolation of chloroplasts.

Leaf pieces were first subjected to vacuum-infiltration of a treatment solution (either distilled water or a herbicide solution) until they sank. They were then transferred to a large surface container (2 liter beaker or large Petri dish) with the treatment solution supplemented with 10 mM bicarbonate at pH 7.0, and irradiated from both above and below with two 100 watt incandescent lamps for 2 hours.

Chloroplasts were isolated from these leaf pieces using the same procedure as employed during the preceding year (CHAPTER III of the Final Report of the preceding year).

Isolation of Mitochondria from Mung Bean Hypocotyls. Mung bean seedlings were grown and mitochondria were isolated from the hypocotyls, by the method described in CHAPTER II of the Final Report of the preceding year.

Activity Measurements. Biochemical activities of isolated mitochondria and chloroplasts were carried out in the same manner as described previously (CHAPTERS II and III of the Final Report of the preceding year), using an oxygen electrode.

Electron Microscopic Study. Mitochondrial suspensions were treated with diallate or DMSO (solvent for diallate) in the manner described in the text. Mitochondria were pelleted at 10,000 x g for 15 minutes at 0-4° C, and subjected to fixation, post fixation, dehydration, embedding, and thin-sectioning using a conventional procedure (cf. CHAPTER VII of the Final

Report of the preceding year). It should be mentioned that the reaction medium for mitochondrial reaction (0.5 M mannitol, 0.01 M potassium-phosphate buffer, pH 7.2, 0.01 M KCl, and 0.005 M $MgCl_2$) was used for fixation with 2% glutaraldehyde and mitochondria were pelleted by means of centrifugation whenever necessary.

RESULTS AND DISCUSSION

A. RESPIRATORY PROPERTY OF MITOCHONDRIA IN HERBICIDE TREATED ROOTS

Prior to the use of herbicide, many attempts were made with soybean roots without herbicide treatment to obtain one or two fractions on the gradient of 'pure' mitochondria which show a high degree of coupling (cf. Douce et al., 1972). Similar to the last year's effort with corn roots, however, we could not succeed to obtain the so-called "tightly-coupled" mitochondria with either differential or gradient centrifugation methods. The problems appear to be associated with the difficulty in the grinding step; less grinding resulted in very low yield and hard grinding led to breakage of mitochondria. Stringy, but hard soybean roots may be the cause for these problems, as compared with much more fleshy mung bean hypocotyls.

Since the purpose of this study was to examine the quality of mitochondria in herbicide treated roots, repeated failure of mitochondrial isolation made it highly difficult to pursue this project, if we intended to use only the "tightly-coupled" mitochondria. We decided, therefore, to analyze all fractions which contained mitochondrial inner membranes on the discontinuous gradient tube, and to use a cytochrome oxidase assay with the ascorbate/TMPD method (see CHAPTER II of the Final Report of the preceding year) using an oxygen electrode.

When a crude mitochondrial fraction, obtained from 500 g to 13,000 x g differential centrifugation, was placed on top of a discontinuous density gradient of sorbitol, cellular membranes were fractionated into 5 major bands, as diagramed in Fig. VII-1, at the interphases of discontinuous gradient. These fractions were carefully collected with a Pasteur pipette, diluted slowly to 0.5 M sorbitol with gentle stirring in the cold room, and centrifuged into pellets at 20,000 x g. The pellets were resuspended individually in the suspension medium. They were then tested for cytochrome oxidase activity.

After incubation of root tips for 2 hours, bromacil was observed to cause only one effect; i.e., it caused enlarged, donuts-shaped mitochondria in isolated corn root cells without affecting other cellular structures (see CHAPTER VII of the Final Report of the preceding year). For this reason, we focused on the effect of bromacil on the distribution of cytochrome oxidase in the five fractions. The results are summarized in Table VII-1. The major cytochrome oxidase fraction is found in Fraction 3 in the control, while it is shifted to Fraction 2 in bromacil treatment. If bromacil is indeed to cause enlarged, ring-shaped mitochondria, our observations suggest that enlarged, ring-shaped mitochondria would be lighter than regular mitochondria, as detected by the cytochrome oxidase assay. Since bromacil was only inhibitory at 10 mM or higher concentrations for the activity of isolated mung bean mitochondria (CHAPTER II of the Final Report of the preceding year), these results would further suggest that the lowered specific activity of bromacil-treated mitochondria is caused by altered bouyancy of mitochondria.

When DNOC treated roots were subjected to the density gradient fractionation, the resultant five fractions showed specific activities comparable to

Fig. VII-1. Sorbitol gradient centrifugation of mitochondrial fraction. See details in text.

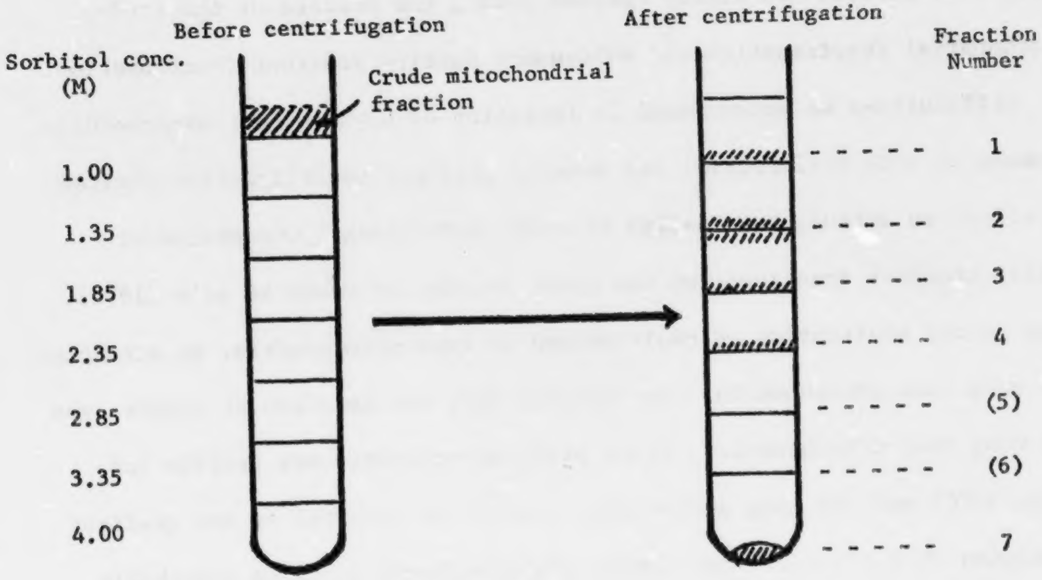

Before centrifugation
After centrifugation
Sorbitol conc. (M)
Crude mitochondrial fraction
Fraction Number
1.00
1.35
1.85
2.35
2.85
3.35
4.00
1
2
3
4
(5)
(6)
7

Table VII-1

Distribution of cytochrome oxidase activity in a discontinuous sorbitol gradient fractions after 10^{-5} M bromacil treatment

Fraction Number	Cytochrome oxidase activity (nmoles O_2/min·mg P)	
	Control	Bromacil
1	9	8
2	279	202
3	513	154
4	171	12
7	92	97
Crude mitochondria before loading	116	107

control values. Since DNOC effects on isolated mung bean mitochondria were reversed by washing (see CHAPTER II of the Final Report of the preceding year), DNOC effects are likely removed during the process of the crude mitochondrial fractionation and subsequent density gradient fractionation.

Difficulties we encountered in isolation of soybean root mitochondria by means of both differential and density gradient centrifugation limited our effort to extend these assays to other herbicides. Our method of density gradient fractionation was based on that of Douce et al's (1972). Prior to our application of their method to root mitochondria, we attempted with mung bean mitochondria. Our results were not as clean as theirs even with mung bean mitochondria. Since plant mitochondria are fragile (cf. Ikuma, 1970) and the long period when sucrose or sorbitol of the gradient is deluted to 0.5 M level may damage the mitochondria, it is remarkable that Douce et al. could obtain clean separation of their fractions and enhanced specific activity. These considerations pose big problems concerning the interpretation of the data. While the data for bromacil effects are fairly clean, the suggestion that enlarged, ring-shaped mitochondria are lighter than normal ovoid mitochondria must be taken with caution at this stage. We need further documentation with improved method of fractionation. Furthermore, it should be added that the formation of enlarged mitochondria as the result of bromacil treatment in soybean root has not been documented in our study. Intact root tips of young soybean plants did not show this bromacil effect (CHAPTER VI) and whether bromacil would cause enlarged, ring-shaped mitochondria in isolated root tips has not been investigated yet.

B. PHOTOSYNTHETIC PROPERTY OF CHLOROPLASTS IN HERBICIDE TREATED LEAVES

Three herbicides were employed in these studies: diallate, diquat, and simazine. These three herbicides caused deformations in chloroplasts when soybean leaf pieces were treated, after infiltration, in light for 2 hours (CHAPTER VII of the Final Report of the preceding year). Diallate and simazine were relatively water-insoluble and 2% DMSO was needed to keep them in solution, while diquat was a highly water-soluble herbicide. Control runs were thus carried out either 2% DMSO or distilled water. Photosynthetic activities analyzed were the same as those in the preceding year's project with isolated chloroplasts : i.e., the non-cyclic photosystem I assay (Asc/DPIP⟶MeV oxygen uptake assay), the non-cyclic photosystem II assay ($H_2O \longrightarrow PD \longrightarrow$ferricyanide oxygen evolution assay), and the non-cyclic assay utilizing both photosystems ($H_2O \longrightarrow$ferricyanide oxygen evolution assay) (see CHAPTER III of the Final Report of the preceding year). For the photosystem I assay and the assay for two photosystems were carried out in the presence or absence of an uncoupler, methylamine.

The results summarized in Table VII-2 show that (a) all herbicides tested act to lower the rates of the activities of photosystem II and both photosystems, but not the rates of photosystem I activity, (b) uncoupled rates by methylamind are about 2 fold higher regardless of herbicide treatment than coupled rates, (c) the photosystem II rates are nearly 3 fold higher than the rates of ferricyanide reduction using both photosystems in the presence or absence of herbicide, and (d) 10^{-4} M concentrations caused expectedly stronger inhibition than 10^{-5} M.

When isolated chloroplasts were treated with a herbicide, diallate and simazine inhibited photosystem II activity and ferricyanide reduction by both photosystems, but not photosystem I activity; diquat did not

Table VII-2

Effects of three herbicides on photosynthetic activity of chloroplasts isolated from herbicide-treated soybean leaves.

Approximately 5 grams of soybean leaves were cut into small pieces, vacuum-infiltrated with herbicides, placed in light for 2 hours, and subjected to isolation of chloroplasts by our standard procedure. Isolated chloroplasts were tested for non-cyclic activities of photosystem I and II singly or in combination, in the presence or absence of an uncoupler, methylamine. The combined activity of two photosystems was examined by the $H_2O \rightarrow$ ferricyanide method; that of photosystem II by the $H_2O \rightarrow PD_{ox}$ method; and that of photosystem I by the DPIP → MeV → O_2 method. The photosystem I assay was an O_2 uptake assay, while the rest was O_2 evolution assays. Each entry is an average of 2-4 determinations.

Herbicide	Conc. used	Activity (μmoles O_2/hr·mg Chl)				
		PS I & II	PS I & II (Uncoupled)	PS II	PS I	PS I (Uncoupled)
	(M)					
Diallate	0	102	183	278	214	443
	10^{-5}	74	147	217	204	411
	10^{-4}	67	105	161	213	420
Diquat	0	56	98	170	161	330
	10^{-5}	43	71	142	147	320
	10^{-4}	34	72	102	157	376
Simazine	0	66	119	192	179	332
	10^{-5}	63	125	212	172	378
	10^{-4}	33	96	160	112	285

inhibit any photoreactions (CHAPTER III of the Final Report of the preceding year). Thus, the results with chloroplasts isolated from diallate- and simazine-treated leaves are more or less expected, while diquat experiments yielded unexpected results from this respect. Diquat, however, caused a strong inhibition of CO_2 uptake with respect to O_2 evolution in the leaf disk photosynthesis study (Table V-2 of the Final Report of the preceding year) and also it caused a strong deformation of chloroplast lamellae (Table VII-2 of the Final Report of the preceding year); its effect on chloroplasts may be more severe in the leaf than the in vitro assays of isolated chloroplasts might indicate. Diallate was found to bind stoichiometrically with organelle membranes, but not diquat and simazine (the Final Report of the preceding year). From the point of view of membrane binding capability of herbicides, the results with diquat and simazine are unexpected.

Since the observed results show some dependency on concentrations of herbicides and methylamine uncouples the basal rates of herbicide-treated chloroplasts in the same manner as control chloroplasts, it is likely that the chloroplasts isolated from herbicide-treated leaves contained mixed populations of damaged and undamaged chloroplasts as the result of treatment. The same argument may also apply to the control chloroplasts; the rates of control chloroplasts vary widely from experiment to experiment. In a given experimental series for a given herbicide, i.e., control and two concentrations of herbicide, the same batch of leaves were used and all necessary treatments were done in an identical manner. Thus the three control runs were made at separate times. Nevertheless, the large variation observed in control rates may indicate different populations of damaged and undamaged chloroplasts.

These considerations posed a question of validity of the results obtained. While herbicidal effects can be explained in terms of the data and available information, large experimental variations pose an unexpected worry, in particular when one is interested in comparing the degree of damage in organelle activity. We have, thus, not proceeded to separate organelle populations in the chloroplast work.

C. DIALLATE EFFECT ON ULTRASTRUCTURE OF ISOLATED MUNG BEAN MITOCHONDRIA

One of many possible modes of action of herbicides on plant metabolism is that a herbicide may act to alter physico-chemical property of cellular and subcellular membranes. Such a possibility was hinted in our work of the preceding year. If, indeed, a herbicide causessuch changes, it may be possible to observe an effect on alteration of membrane structure. A herbicide which binds irreversibly and stoichiometrically with subcellular membrane amy cause such an effect which can be detected by electron microscopy. Although this work was not proposed initially and particularly when we consider technical and interpretative difficulties in organelle population studies, we thought it highly interesting if such an effect was observed. We, therefore, carried out a preliminary series of experiments on diallate effect on isolated mitochondria.

Mitochondria were suspended in the reaction medium and treated with diallate for 10 minutes at room temperature (state 1 mitochondria). Another run was made with state 4 (substrate + ATP) and treated with diallate for 10 minutes at room temperature. Controls were run with appropriate amounts of DMSO, the solvent of diallate. Mitochondria were pelleted by centrifugation at 10,000 x g for 15 minutes at 0-4^{o} C, and prepared for electron microscopic observations using the standard, conventional procedure

(MATERIALS AND METHODS).

The control mitochondria, either state 1 (Fig. VII-2) or state 4 (Fig. VII-3), show expected ultrastructure of outer and inner membranes. However, diallate-treated mitochondria (Figs. VII-4 and VII-5), regardless of respiratory states they were in, are (a) sticking each other, (b) lacking the outer membrane, (c) showing less number of cristae, and (d) cristae are enlarged, instead of thin, healthy appearance..

In these experiments, 2% glutaraldehyde was added to the pelleted mitochondria. If the fixative (2% glutaraldehyde) was added before centrifugation to obtain the mitochondrial pellet, the dramatic sticking phenomenon does not take place (Fig. VII-6 and VII-7). The sticking phenomenon was observed only when the mitochondrial pellet was washed free of the bulk of diallate by addition of 2% glutaraldehyde to the pelleted mitochondria. The mitochondrial sticking phenomenon was detected in a diallate concentration range of 50 μM - 500 μM.

Since the sticking phenomenon is an outer membrane phenomenon, yet diallate effect could be detected in electron transport (see CHAPTER II of the Final Report of the preceding year), it is of interest to examine if the inner membrane of the mitochondrion would respond in the same manner as the whole mitochondria. For this purpose, mitochondria was osmotically lysed and the inner membrane (ETP) fraction was collected. To the EPT fraction, diallate or DMSO was added in the manner to stimulate sticking. There is no sticking phenomenon observed whether the ETP was treated with DMSO or with diallate.

It thus appear that diallate does not exert any effect on the inner mitochondrial membrane morphologically, but it apparently alters the physicochemical characteristics of the outer membrane. Diallate-induced sticking phenomenon is dependent how diallate and the fixative were administered.

Fig. VII-2. (Top) DMSO treated mitochondria at respiratory state 1.

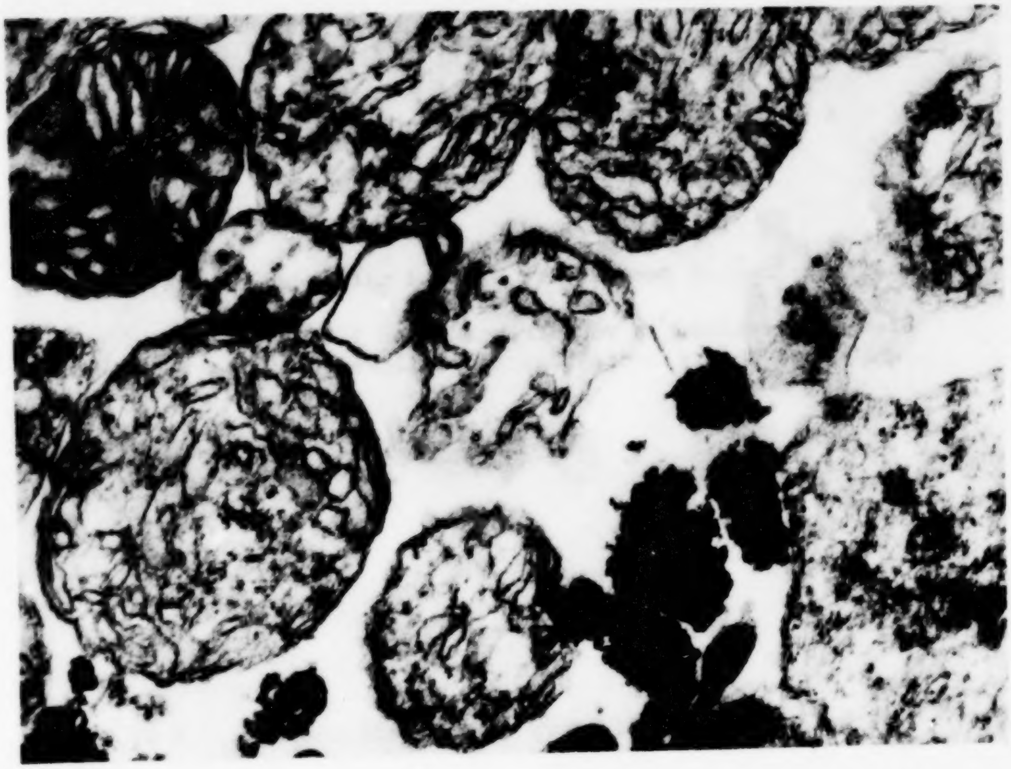
X 61,500.

Mitochondria were treated with 1% DMSO for 5 minutes.

Fig. VII-3. (Bottom) DMSO treated mitochondria at respiratory state 4.

X 61,500

Mitochondria were brough to state 4 with successive additions of 30 mM malate and 140 μM ATP. 1% DMSO was added for 5 minutes after 5 minutes from ATP addition.



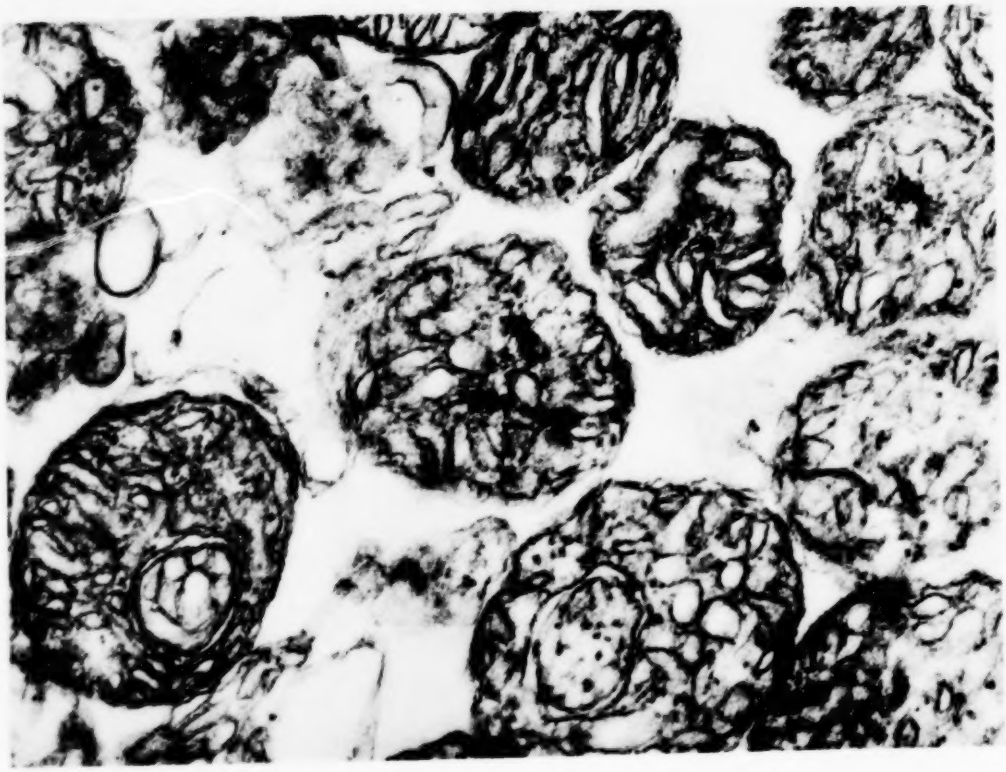
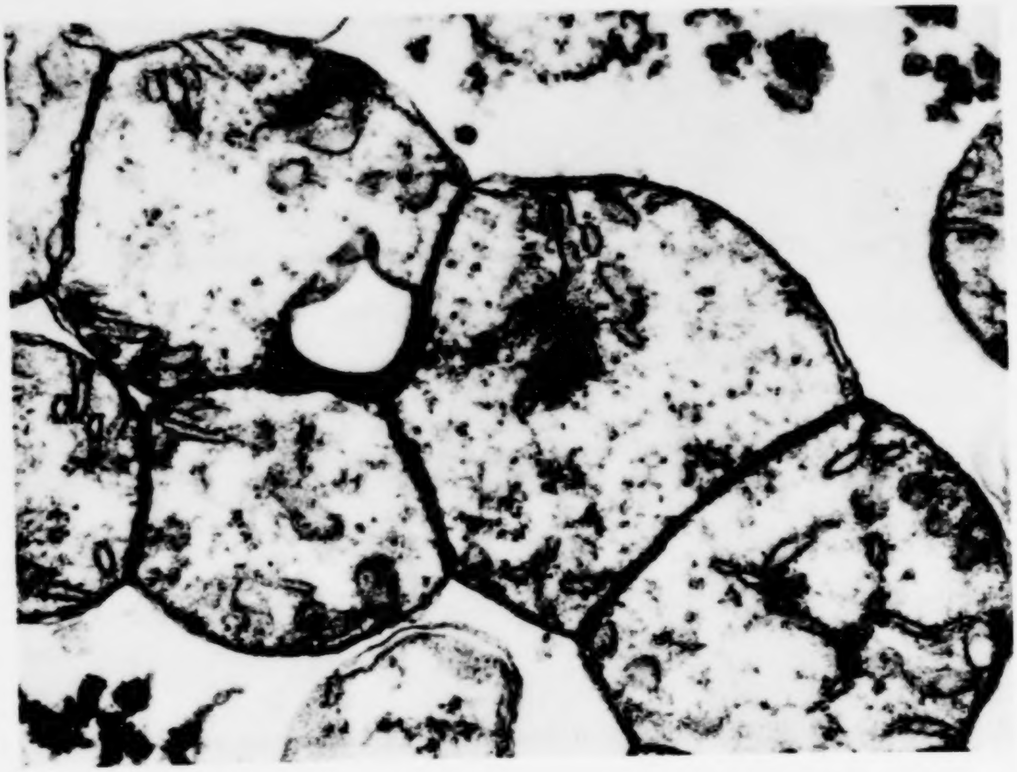


Fig. VII-4. (Top) Diallate-treated respiratory state 1 mitochondria. X 61,500.

State 1 mitochondria were treated with 100 µM diallate for 5 minutes, before centrifugation followed by glutaraldehyde fixation.

Fig. VII-5. (Bottom) Diallate-treated respiratory state 4 mitochondria. X 61,500.

Mitochondria were brought to state 4 by successive additions of 30 mM malate and 140 µM ATP. 5 minutes after the ATP addition, 100 µM diallate was introduced for 5 minutes. The mitochondria were pelleted by centrifugation and the pellet was fixed with 2% glutaraldehyde.



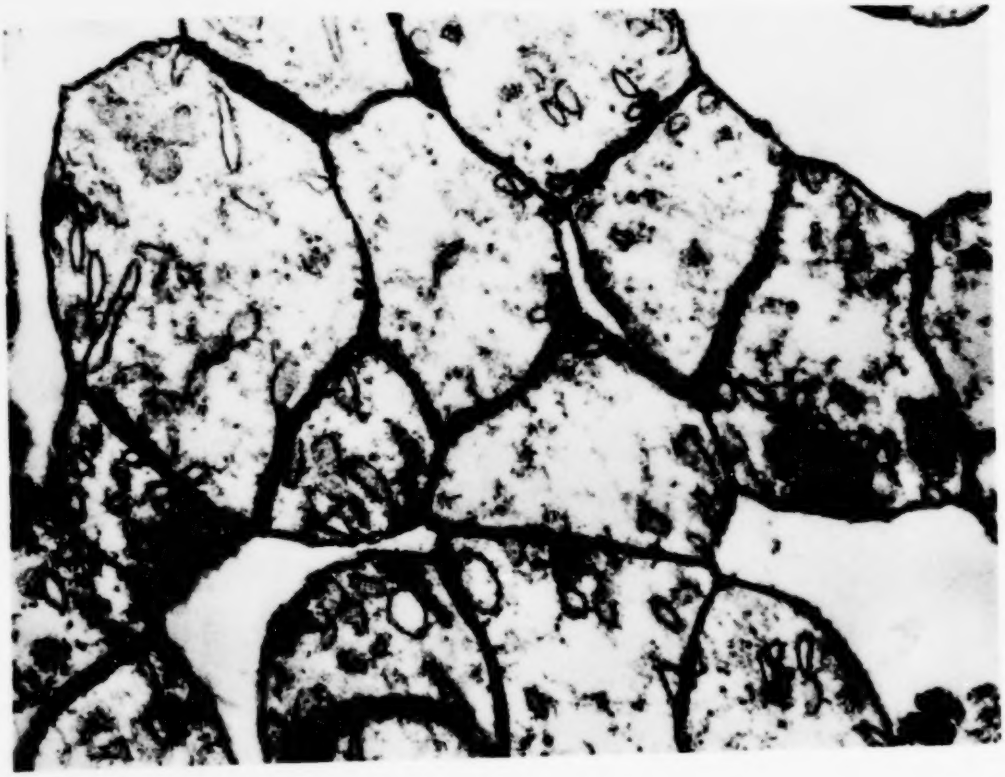
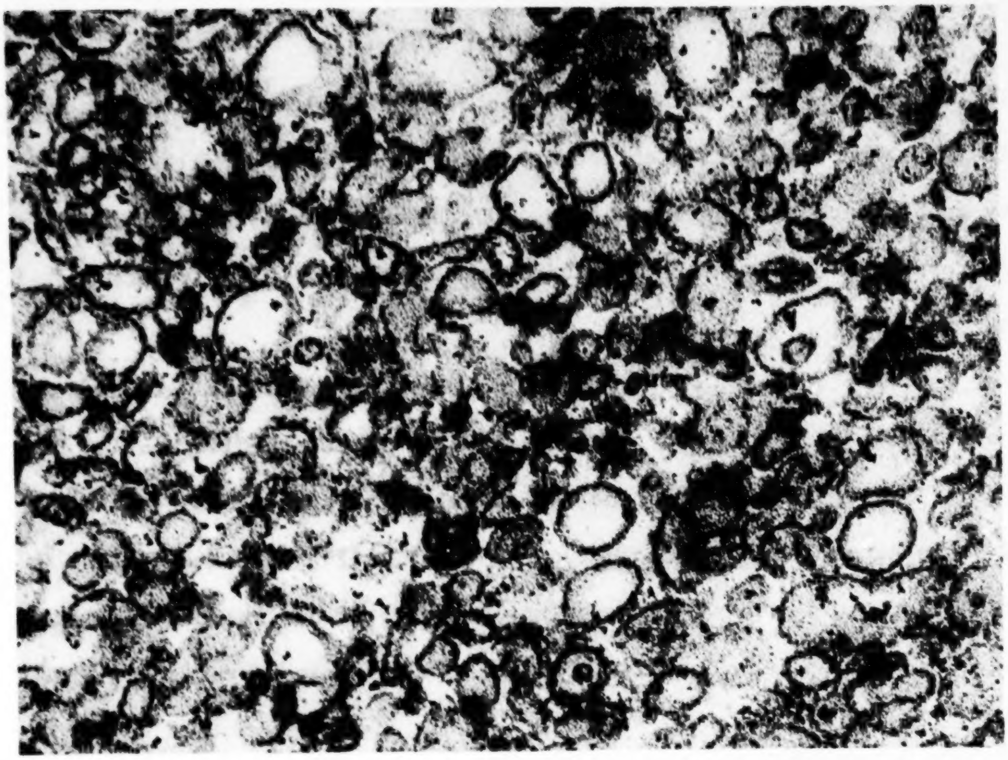
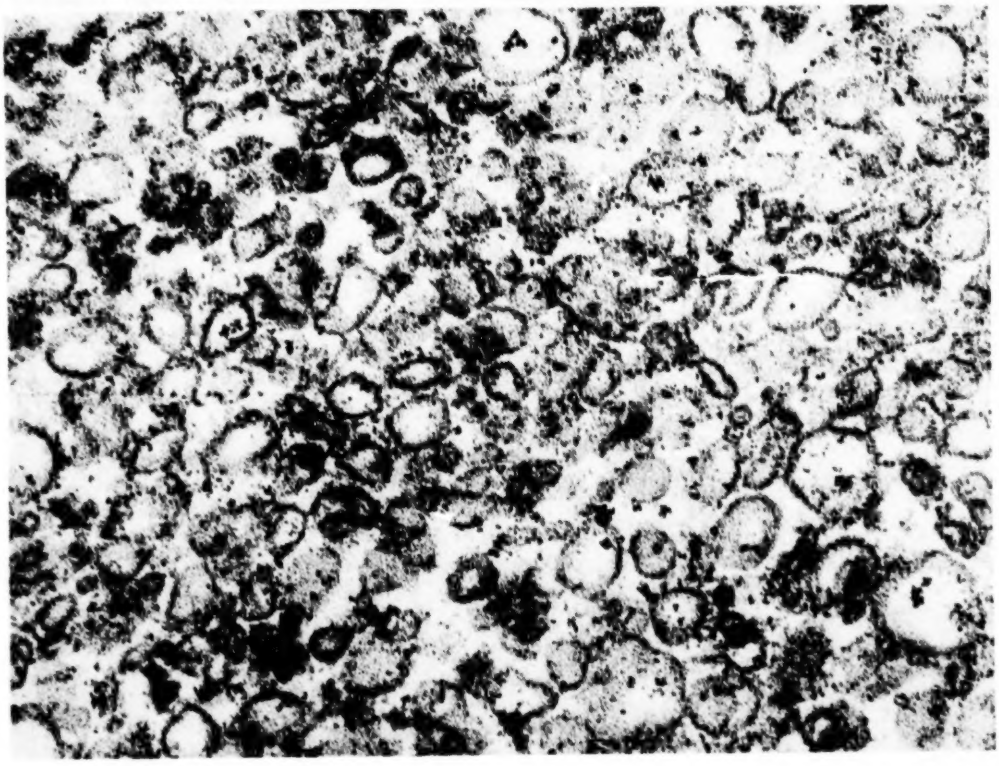


Fig. VII-6. (Top) ETP fraction of mitochondria incubated with 0.33% DMSO for 15 minutes. X 119,700.

The submitochondrial ETP (electron transport particle) fraction was obtained as follows: mitochondria were first lysed in hypotonic medium, the 20,000 x g pellet was sonicated for exactly 60 seconds, and the 100,000 x g pellet was collected as ETP.

Fig. VII-7. (Bottom) ETP fraction of mitochondria incubated with 100 uM diallate for 15 minutes. X 119,700.





EVALUATION OF METHODS

Two major premises and two major methods were employed in these studies. The first was the examination of the so-called abnormal organelles using a density gradient centrifugation method, and the second was the examination of the properties of organelle membranes as detected in ultrastructural alterations.

For the first type of work, mitochondria and chloroplasts were isolated from herbicide-treated tissues. The results from these studies were at best ambiguous; problems centered around the experimental techniques were not solved satisfactorily. The second type of study, using diallate effects on ioslated mitochondria, provided a definitive results, but whether such a technique can be applied routinely to herbicide screening work is still a question.

Although the results in this chapter have not been clear, such results can be viewed as instructive in that the techniques employed in these studies are still premature to use for herbicide screening work.

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